THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXII NOVEMBER–DECEMBER, 1956 NUMBER 6

BALANTIDIASIS

A REVIEW AND REPORT OF CASES *

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Balantidium coli is probably the rarest pathogenic organism causing dysentery. A review of the literature shows that about 600 cases have been recorded in which this protozoon was the sole or main parasite causing colitis. It was in 2 cases of dysentery that Malmsten, in 1857, discovered the parasite in the stools in human cases and named it *Paramecium coli.* In 1863, Stein renamed it *B. coli.*

GENERAL CONSIDERATIONS

Geographic Distribution

The organism has been found as far north as Finland, Sweden, and Norway, but occurs most frequently in subtropical and tropical zones. In the Western Hemisphere, it has been identified in Canada, the United States, Mexico, Honduras, Costa Rica, Panama, Jamaica, Cuba, Puerto Rico, Venezuela, Colombia, Brazil, Uruguay, Argentina, and Chile. It is, however, world-wide in distribution. Large numbers of cases have been reported from Brazil, Mexico, the Philippines, Persia, and the United States; 61 cases had been observed in the United States up to 1950.

Incidence

The reported incidence of parasitization varies enormously, most probably depending on the methods of investigation. It is generally accepted that the parasite is found in about 0.07 per cent of all stools examined, but there are good reasons to believe that this figure falls short of the real one. Stshenovitsh, working in Khalnar, Azerbaijan (Persia), found an incidence of 5.1 per cent among 2,000 fresh stool specimens, in contrast to a study conducted in the same area a few

* Received for publication, December 6, 1955.

years earlier, which yielded only 0.02 per cent. The discrepancy is attributed by Stshenovitsh to his predecessor's failure to examine freshly collected specimens. Stewart, in Southern Persia, discovered 17 cases among 1,430 cases of dysentery. Two of these were complicated by the Flexner type of Shigella and the other 2 by the Sonne type. Kipschidse observed 22 cases over a 4-year period in Tiflis, and McCarey recorded 87 cases over a 9-month period, also in Southern Persia. Burrows and Jahnes noted an incidence of 1.3 per cent among 544 patients in the Insular Penitentiary of Puerto Rico; of these cases, 332 were inmates, among whom the incidence of parasitization was 1.8 per cent. Most other authors give lower figures, as shown in Table I.

The lower values correspond to series in which no data were given as to how soon after evacuation the stools were examined. In regard

TABLE I

Reported Incidence of Balantidial Infection

Author	Country	No. of cases	Positive	Contact with pigs
			%	
Stshenovitsh*	Persia (Azerbaijan)	2,000	5.1	0
Stewart	South Persia	1,430	0.01	0
Burrows and Jahnes*†	Puerto Rico	544	1.3	+
Atiles	Puerto Rico	5,000	0.08	+
Serra	Puerto Rico	5,000	0.08	+
Maldonado	Puerto Rico	8,000	0.03	+
Cort	Siam (Chiengmai)	8,000	0.17	+
Sommerville	Argentina	5,000	0.02	+
Bowman (1909)	Philippine Islands	4,000	0.07	+
Pawel	Brazil (Saõ Paulo)	12,500	0.02	+
Maia	Portugal	4,000	0.1	+
Potenza and Martínez	Venezuela (Caracas)	5,689	0.05	+
Young (1939)*†	United States (South Carolina)	142	4.9	0
Ferri*†	Russia (Tiflis)	68	29.0	+

* Fresh stools.
† Institutional cases.

to cases from Puerto Rico, it can be safely stated that most of the stool samples were examined many hours after passage. In Maldonado's series the specimens were received in the Department of Parasitology of the School of Tropical Medicine 2 or 3 days after evacuation, well beyond the period considered optimal for identification of the trophozoites. It seems to us that the discrepancy in incidence results mainly from rapid disintegration of the parasite in the

stools after evacuation. Maia and others have observed that 6 hours after the passage of the stools few or no trophozoites can be identified. Furthermore, it is characteristic of this condition that the balantidia are passed intermittently, so that they may be abundant one day and absent 2 or 3 days later. This must be kept in mind when conclusions are drawn as to the effectiveness of therapeutic agents.

Morphology

The parasites are readily identified, for they are the largest protozoa infecting man. In the trophozoite (Fig. 1) stage they are ovoid and vary from 30 to 150 μ in length and from 40 to 55 μ in width, although specimens as long as 300 μ have been seen. The parasite is ovoid and in its anterior portion, slightly lateral to the midline, there is a funnel-like depression, the peristome, communicating with the cytostome and the cytopharynx. The body is enveloped in a thin pellicle covered by numerous cilia that extend over the primitive buccal slit; and it is by their action that the organism is capable of very active locomotion and that food is directed into the digestive passages. At the posterior end there is a small, hardly visible, triangular opening, the cytopyge, with an excretory function. The cytoplasm contains two contractile vacuoles, and often red blood cells, starch granules, oil droplets, and inert materials. The characteristic macronucleus, which is shaped like a kidney or bean, is slightly eccentric; in its concavity a tiny micronucleus is seen occasionally. The macronucleus appears as a homogeneous mass of chromatin, staining evenly and deeply with basic dyes.

The cystic (Fig. 2) stage is characterized by a fairly large, round or ovoid structure, 40 to 65 μ in diameter. In fresh, non-stained preparations it has a dirty, opaque grayish color and presents bar-like bundles of inclusion material which is colorless and disappears on staining. It must be kept in mind that in fresh preparations the macronucleus is not visible. By using Delafield's or Heidenhain's hematoxylin the characteristic macronucleus, the granular cytoplasm, and the absence of stomata and cilia can be brought out. When the parasite is in the process of encystment, cilia may be observed under the capsule, and the organism is seen rotating actively within this capsule.

Nutrition

For their nutrition balantidia depend mostly on starches and red blood cells. Being very susceptible to the action of acids, they are quickly destroyed by the gastric juice of a normal person. Many methods of culture of the organism have been devised. Barret and

Yarbrough used 1 part of inactivated human serum and 16 parts of 0.5 per cent sodium chloride, giving a faintly alkaline reaction to litmus paper. The medium is placed in test tubes 10 by 150 mm. in size, and incubated at 37° C. for 24 hours. Dobell and Laidlaw used inspissated horse serum and Ringer's fluid-egg white with solid rice starch at a pH of 5 to 8, keeping the cultures at 37° C.; subcultures were made every 3 to 5 days. Although conjugation was observed periodically under these conditions, encystment did not occur. Schumaker (1931) used potato, corn, wheat, and arrowroot starch with equally good results, and noted that temperatures below 34° C. were harmful to the organism.

The parasite is a facultative anaerobe but it grows well in both aerobic and anaerobic media. Nelson used intestinal contents freed of coarse particles by filtration and proved that balantidia could obtain from such contents all the essentials for growth and multiplication; serum need not be added, but the normal bacterial flora of the intestinal contents was indispensable for their growth. A pH below 5 is incompatible with the life of the balantidium. Despite the high sensitivity of the ciliate to a low pH, Hegner (1926) was able to recover live trophozoites from the colons of guinea-pigs after ingestion. A diet rich in protein will decrease their growth in the colon. Schumaker (1930) found that rats maintained on a casein-rich diet (70 per cent), were prevented from developing the infection; when the diet was changed to 93.5 per cent carbohydrates, multiplication of balantidia was greatly enhanced, and the proportion of infected rats was greatly increased. Van der Reis (1923) and Schumaker (1931), independently, studied the intestinal flora of heavily parasitized rats and noted the predominance of *Lactobacillus acidophilus*, while lactose fermenters were very much diminished. The opposite was true when the balantidia were scant or absent. This compares with the studies of Sommerville in cases of balantidiasis, in which he noted a neutral or faintly alkaline reaction of the stools.

Metabolism

Schumaker (1931) observed that the parasite was unaffected by oxygen pressure of 32 lbs. per square inch for 72 hours. Daniel determined that the respiratory quotient of the parasite is 0.84, indicating that under aerobic conditions carbohydrates are not the chief source of energy for balantidium. Agosín and von Brand recently have studied its respiratory metabolism and have concluded that the aerobic gaseous exchange is characterized by a fairly high rate of oxygen

consumption, with respiratory quotients of slightly above 1.0. Anaerobically, relatively large amounts of CO_2 are given off. Aerobic and anaerobic gaseous exchanges were relatively insensitive to DL-glyceraldehyde but sensitive to glycolysis inhibitors, anaerobiosis being more sensitive to lower concentrations of a given inhibitor than the aerobic phase. Inhibition by malonate and fluoro-acetate suggested an aerobic sequence showing some characteristics of the Krebs cycle. Glaessner isolated a glycolytic enzyme and a hemolysin from the parasite, but was unable to demonstrate any proteolytic enzyme.

Epidemiology

The protozoon has been found in the hog, wild boar, sheep, horse, bovines, guinea-pig, fowl, turtle, cockroach, *Macaca mulatta,* orang-outang, baboon, chimpanzee, and other species. However, of all animals in relatively close contact with man, the hog is the one that is more frequently and most heavily parasitized. Ostroumov, in Central Soviet Russia, found 62 per cent of the pigs heavily infected; Shegalow, in St. Petersburg, 40 per cent; Kipschidse, in Tiflis, 63.3 per cent; and Füsthy, in Szeged, 92 per cent, and in Budapest, 90 per cent. Walker, Liu, Shun-Shin, Pawel, Cruz and Domingo, and others referred to the heavy rate of infection among hogs in the countries from which they reported. Twenty-five per cent of the cases give a history of contact with hogs, and Selimkhanov and Ferri, among others, believed that there is an important relationship between the heavy infection of hogs and human cases. Our review of the literature shows that more than 50 per cent of cases give a history of contact with pigs. On the other hand, Liu, Shun-Shin, and Shegalow remarked that despite the heavy infection of hogs in their countries the number of human cases was very small. The relationship between man and hog also is rendered doubtful by McCarey's series; being Mohammedans, his patients did not eat pork nor live in close contact with pigs. In Young's series (1939) there was no history of contact with hogs, and Awakian remarked that although the number of hogs is high in England, cases of human balantidiasis are extremely rare, and that in Armenia, where hogs are scarce, balantidiasis often affects man. Tzaturian observed 24 cases in 7 years, of which only 2 patients admitted contact with pigs.

All of these controvertible observations have led some authors to believe that there is a difference between the human and porcine balantidia; the former has been called *B. coli* and the latter *B. suis.* Hegner (1934), Selimkhanov, Walker, and Pritze, among others,

despite extensive and painstaking studies, have been unable to differentiate one from the other on a morphologic basis. Differentiation on the basis of size alone is fallacious, since balantidia may vary enormously in size, probably depending on the dietetic habits of the host. Chichulin reported on a family, all members of which developed balantidiasis after eating raw hog sausage, presumably heavily parasitized. Awakian found 29 per cent of the rats in Southern Russia infected with the ciliate, and Klein discovered it in the sewer system of London. The parasite also has been observed in the diarrheic stools of monkeys and chimpanzees, and in a capybara. Tsuchiya and Kenamore attributed an important rôle to flies in the transmission of their case.

There must be other factors to explain the irregular incidence of balantidiasis. Worthy of note is the fact that the more severe infestations have been reported from underprivileged areas where standards of hygiene and nutrition are inadequate. Furthermore, the only epidemics have been reported from mental institutions where the patients were untidy, negativistic, or even coprophagic (Young, 1939; Ferri). Elliott and Hotson, and Junqueira also mentioned coprophagia among their patients.

Pathology

Pathogenicity

Some believe that *B. coli* is completely harmless. Others think that it is pathogenic only on tissues previously damaged otherwise, as by bacteria. Still another group considers that the ciliate is pathogenic per se. The natural resistance of man to this parasite is evidenced by the persistent failure by Young (1950) and Knowles and Das Gupta to transmit the infection to human volunteers. On the other hand, Walker was able to reproduce the disease experimentally in monkeys by feeding and by rectal inoculation with trophozoites from pigs. Brumpt infected monkeys with material obtained from pigs, and vice versa. Harms was unable to induce the disease in pigs with trophozoites from a human case. Hegner (1926) recovered live balantidia from the colons of guinea-pigs that had ingested trophozoites.

Morphologic Features in Man and Experimental Animals

The number of cases studied histopathologically is small. The first large series published was that of Strong, who in 1904 collected 40 cases from the literature and added 7 of his own. After that only isolated reports appeared. The most recent series came from this laboratory in 1947, when Koppisch and Wilking described the necropsy findings in 4 cases.

Balantidiasis usually involves the colon (Fig. 4). Grossly, the lesions involve the large intestine from cecum to rectum, although the rectosigmoidal segment is the region more commonly and more severely affected. The earlier lesions appear as small, flask-shaped ulcers a few millimeters in diameter. Later these lesions expand into ulcers resembling those of amebic colitis. They may be numerous or scarce; occasionally they replace almost the entire colonic mucosa. The edges of these ulcers are frayed, ragged, swollen, and frequently undermined. They are covered with mucous material or with a necrotic, grayish white, slate black, or black membrane. The mucosa about the ulcers may be reddened and swollen, or may appear practically normal. The ulcers are for the most part superficial, but some may affect the whole thickness of the intestinal wall, so that perforation may occur. After perforation, the omentum may be plastered over the involved segment or a generalized peritonitis may result. Although the ulcers usually stop at the ileocecal valve, in some cases the process may extend to the distal portions of the ileum, as in 2 cases seen by Koppisch and Wilking.

The earlier lesions, as described by Walker in the experimental animal, appear as zones of slight hyperemia of the mucosa with or without punctiform hemorrhages. Foci of vascular dilatation and perivascular round cell infiltration associated with eosinophils are seen also. The absence of polymorphonuclear leukocytes and the abundance of round cells and eosinophils is a characteristic that distinguishes balantidiasis from the bacterial infections. The balantidia penetrate through the intact epithelium of the colon, chiefly that of the crypts. At the site of contact between the ciliate and the epithelium, the cells may become shrunken (Fig. 3), the cytoplasm turning markedly acidophilic and the nucleus pyknotic. The glandular epithelium may be thinned out. Whether entering directly through the surface epithelium or through glands, the parasite usually penetrates the basement membrane and then the muscularis mucosae, to reach the submucosa (Fig. 5). At this level it often is found in dilated lymphatics (Fig. 6). The lymph vessels and small capillary blood vessels are then much dilated. The mucosa and submucosa undergo necrosis, and the limiting portions become infiltrated chiefly with lymphocytes, although in places neutrophils may be numerous (Fig. 7). Foci of hemorrhage are frequent, and congestion is very prominent.

When the overlying mucosa is cut off from its blood supply, necrosis and ulceration follow. The ulcers are irregular and have undermined edges (Fig. 8). They are partly covered by a coagulum of fibrin, red blood cells, leukocytes, and cellular débris (Fig. 9). Occasionally an

abscess is found in the submucosa beneath an intact, overlying mucosa; serial sections will usually demonstrate that this occurs in the vicinity of an ulcer. Balantidia are difficult to identify in the necrotic portions of ulcers, but they are numerous in the periphery.

The organisms are seen also at a distance from the ulcers, in the muscular coat, in the serosa (Fig. 10), and even in regional lymph nodes (Fig. 11). Campos observed that those found in the muscle coat were surrounded by a clear space which separated them from the tissue; however, this is probably the result of fixation and shrinkage rather than of any specific change. In these locations there is very little reaction, if any, about them. Manlove claimed that this is explained by the tendency of this ciliate to migrate post mortem, and his experiments seemed to confirm that impression. In the appendix the lesions are similar to those of the colon, so that no special reference is necessary (Fig. 12). No lesions have been reported as occurring in either regional lymph nodes or in the liver.

Clinical Features

The presence of balantidium in the feces is not always an indication of disease. Swartzwelder classified the clinical picture as follows: (1) asymptomatic, the main danger of this form being the rôle these patients play as carriers, chiefly in mental institutions or penitentiaries; (2) a chronic form with intermittent episodes of diarrhea; and (3) the dysenteric form, which may be mild, severe, or fulminating.

The *chronic form* is characterized by the presence of loose bowel movements alternating with episodes of constipation. There may be epigastric distress, colicky abdominal pain, and tenesmus. The number of bowel movements varies from 3 to 20 a day and mucus often is seen, but pus and blood only rarely. Loss of weight is moderate, but may be marked when the course is protracted. Balantidia are identified in the stools only sporadically, so that repeated examinations and the study of fresh stools are mandatory.

The *acute form* appears suddenly with 3 to 15 bowel movements daily, accompanied by tenesmus; the stools contain mucus, blood, and neutrophils. The patient complains of epigastric distress, nausea, and abdominal pain, with tenderness along the colon. Loss of weight may be rapid, some patients having lost 40 kg. over a period of 3 months. Weakness is marked and is related to dehydration and undernourishment. Swartzwelder claimed that these patients have a peculiar odor to their breath, recalling a "pigpen." Proctoscopic examination reveals diphtheritic patches, 1.5 to 3 cm. in diameter, surrounded by a bright

red, swollen mucosa. Occasionally tiny ulcers also are seen, requiring differential diagnosis from bacillary or amebic dysentery. Balantidia may be extremely numerous in the scrapings of such ulcers. Pallor and hypochromic anemia may result from hemorrhage and undernourishment. Some authors have attributed a megaloblastic anemia to the parasite, but these patients either suffered from sprue or the gastric juice showed achlorhydria, so that pernicious anemia could not be ruled out. Leukocytosis and eosinophilia are not found unless there are complications such as perforation or association with other parasites, such as Uncinaria.

In the *fulminating type,* seen ordinarily in emaciated patients, or in the late stage of some other severe disease, diarrhea starts suddenly with from 5 to 25 bowel movements a day. Overt hemorrhages may occur, leading to death by exsanguination; otherwise there is dehydration and rapid deterioration, with death in 3 to 5 days. Although most cases are afebrile, temperatures as high as 38.1° C. have been observed in the chronic and dysenteric forms. The chronic form may last for years; McEwen has reported a case of 20 years' duration.

Balantidial Appendicitis

Jaffé and Kann (1943), from Venezuela, were the first to describe balantidial appendicitis. They reported 6 cases, in 5 of which the complaints were of chronic nature and in the sixth there was a sudden onset of abdominal pain, shown at operation to be due to acute appendicitis. Jaffé and Kann were unable to identify balantidia in the appendiceal walls of the 5 chronic cases, but they were abundant in the lumina. In the other case balantidia were seen throughout the wall, and the ciliate was considered the cause of the appendicitis. In 1947, Potenza and Martínez, also from Venezuela, reported the second case of acute appendicitis due to *B. coli.* The patient was a child who developed abdominal pain in the right lower quadrant, with positive McBurney and Rovsing signs, as well as neutrophilic leukocytosis. To the best of our knowledge, these are the only cases of acute appendicitis ascribed to the *B. coli* that have been recorded. Worthy of note is the fact that repeated examination of the stools for a period of 6 months after the operation failed to reveal any cysts or trophozoites.

Diagnosis and Therapy

The diagnosis is based on the identification of the parasite in the stools or in scrapings obtained at proctoscopy. Any dysenteric process ought to include also a careful search for amebae and dysentery bacilli;

combinations of these diseases sometimes occur. Overlooking another parasite by the identification of only one species may lead to grave consequences for the patient. The material obtained must be examined immediately, keeping in mind the rapid deterioration of the trophozoite. Cysts are seldom found; Young (1939), among 142 examinations, observed trophozoites in 87.5 per cent and cysts in only 8.9 per cent. Ferri found no cysts in his cases. The material may be examined by making a saline suspension or after staining with Delafield's or Heidenhain's hematoxylin. As the ciliate is large, the use of a low-power lens usually is adequate; however, considering the enormous variations in size, a more careful search should be made at a higher magnification if low-power examination proves negative. The parasite has been identified in the urine, although only once, by Maliwa and Haus who later demonstrated the organism in sections of ureter and bladder in the same patient. Stokvis discovered the parasite in the sputum of a patient supposed to have a liver abscess perforating through the diaphragm into the lung; however, this supposition was never proved. Finally Hinkelmann claimed to have encountered *B. coli* in the blood of a patient who also showed it in the urine, but this case is open to question.

The prognosis of balantidiasis, especially for the acute and fulminating types, was very poor until 1950, with a mortality of up to 30 per cent. The introduction of antibiotic drugs has improved the outlook. The number and varieties of drugs and treatments previously used speak for the poor results and inefficacy of most. Greene and Scully, in 1923, used a starch-free diet consisting of 2½ quarts of milk a day, to which soft eggs were added. Silva also obtained good results in the cases thus treated, but such measures have failed in the hands of others. Cort, after the unsuccessful use of emetine, neoarsphenamine, and acetarsone, gave his patients 15 ml. of oil of chenopodium in 150 ml. of olive oil per rectum, obtaining complete cures in all cases. Serra treated a patient of his following the same technique, and the patient died of oil of chenopodium poisoning within a few hours. Kipschidse considered emetine the best drug, but Cruz and Domingo, and DeLanney and Beahm got no results with it. Iodine compounds, either alone or in combination with other drugs, have been used successfully by some authors (DeLanney and Beahm, Swartzwelder, Young and Walker, Pramanik) while others had complete failures (McCarey, Cort). Of all the chemotherapeutic agents, the arsenicals, such as stovarsol, carbarsone, acetarsol, tryparsamide, neoarsphenamine, and spirocid, gave consistently good results. Carbarsone, alone

or in combination with iodine derivatives, was considered the best. Sulfaguanidine and other sulfa derivatives also have been prescribed with contradictory and irregular benefits. As complementary therapy, enemas of methylene blue, yatren, protargol, and quinine sulfate also were employed.

In 1950, Castellanos *et al.* reported on the excellent results obtained with bacitracin in two children with balantidial dysentery. In the same year Agosín *et al.* published their observations on the action, both *in vivo* and *in vitro*, of aureomycin and terramycin against balantidia; they attributed this action not to any change in the pH or in the intestinal flora but to a direct effect of the drug on the parasite. Beneficial results also were reported by Neghmé *et al.* in 1951, who obtained complete disappearance of the parasite from the feces in 2 to 4 days. Later on, Weinstein *et al.*, Jarpa and Allende, and Hoekenga used terramycin, while Santos, Burrows and Jahnes, and Neghmé *et al.* used aureomycin with very good results. The dose of terramycin has been 500 mg. 4 times daily for 10 days and for aureomycin, 2 gm. daily for a total of 28 gm.

Report of Cases

In a previous communication (Koppisch and Wilking) one of us gave examples of two modalities of balantidiasis. Case 1 (necropsy no. 1196) represented a very acute case of balantidial dysentery with involvement of the colon and terminal ileum in a girl 19 years old. Death was due ultimately to two perforations of the cecum and one of the sigmoid colon, with diffuse peritonitis. Case 2 (necropsy no. 1384), a 4-year-old mulatto girl, was an example of the chronic form, with six ulcers distributed along the colon, and with extensive pulmonary and glandular tuberculosis as the main disease.

Case 3

A 7-year-old boy was admitted to the hospital because of severe diarrhea, vomiting, and headache for 3 days. At onset, the patient vomited seven worms (Ascaris), after which he was unable to retain food or liquids. Temperature was 99° F. The patient was poorly developed, poorly nourished, markedly dehydrated, and slightly cyanotic. The pupils were dilated, did not react to light, or in accommodation. He was given 5 per cent glucose in saline solution intravenously, and shortly thereafter became restless, slightly cyanotic, and dyspneic. Râles were heard and in a few minutes apnea supervened. He was placed in a Drinker respirator. The liver was palpable 3 fingerbreadths below the costal margin. The extremities were cold and flaccid and reflexes could not be elicited. The diarrheal stools were greenish and bloody. The urine showed traces of albumin and a few casts. The condition of the patient deteriorated rapidly and he expired 19 hours after admission.

Gross Examination

The body (necropsy no. 2128) was that of a poorly developed, poorly nourished and dehydrated boy. The abdominal cavity contained 200 ml. of turbid yellow liquid with multiple flecks of fibrin. The peritoneum was smooth and glistening. The liver was enlarged and the bladder rose 7 cm. above the pubis. Approximately 75 ml. of yellow fluid was found in each pleural space and there were several fibrous pleural adhesions over the posterior aspect of the lungs. In the right frontal region there was a hematoma, 1.5 cm. in diameter, and another in the occipital region, measuring 1.6 cm. across. The bones of the skull, the meninges, and the surface of the brain were normal. There was an early thrombus in the confluence of the sinuses. The heart was normal. The lungs were markedly congested and edematous. The small intestine showed numerous large, discrete areas of mucosal congestion and several adult Ascaris. The mucosa of the colon showed congestion and tiny ulcers measuring about 1 mm. across. Whipworms were abundant in the rectum, sigmoid, and appendix. The other organs were normal.

Microscopic Description

Heart. There was moderate serous atrophy of the subepicardial fat. A slight diffuse infiltration of the myocardium with lymphocytes and eosinophils was present, together with slight interstitial edema.

Lungs. Marked congestion of the interlobular septa was present. Coagulated protein filled the alveolar spaces. A few macrophages and neutrophils were seen in air sacs and occasional hemorrhages were present also.

Spleen. Congestion and hemorrhages into the splenic pulp were evident.

Liver. Most hepatic cells contained fat droplets. The portal spaces were broadened by fibrosis. The central zones revealed slight congestion.

Adrenal Glands. The adrenal cortex was almost completely depleted of lipids. The zona fasciculata showed focal atrophy. There was an extracortical adenoma.

Ileum. In the ileum edema of the submucosa was accompanied by infiltration with eosinophils.

Colon. Numerous balantidia were found in the colon between the detached mucosa and the basement membrane. Some had migrated into the submucosa and there they had induced a marked infiltration with eosinophils, lymphocytes, and plasma cells. This was diffuse but

in some areas it became more dense. In such regions there were minute foci of necrosis and hemorrhage. Balantidia were found in the margins of these abscesses. The overlying mucosa was thinned but not ulcerated. The blood vessels were filled with eosinophils and lymphocytes and the capillary network of the mucosa also was diffusely dilated and filled with eosinophils. The lymphatic vessels were dilated; in some the endothelium was slightly swollen. About a few lymphatics there were groups of lymphocytes and eosinophils forming nodules that bulged into the lymphatic lumen and narrowed it. Sections from an early ulcer showed numerous balantidia in the mucosa and upper portion of the submucosa, surrounded by lymphocytes and a few eosinophils. A few balantidia were seen also in the mesenteric lymph nodes where they had induced a moderate hemorrhage; one was found in an afferent lymph vessel in the hilum of the node without any reaction about it.

Case 4

An 11-year-old Negro boy (S.P. no. 75066) was admitted to the hospital because of abdominal pain of a few hours' duration. He had felt well until March 31, 1955, when, on awakening, he complained of mild pain in the right lower abdominal quadrant. He vomited shortly thereafter, and the pain, nausea, and vomiting increased in severity. There had been no bowel movement since the onset. He had had no similar attacks in the past. Physical examination revealed the temperature to be 103° F.; pulse, 116 per minute; blood pressure, 115/70 mm. of Hg. The abdomen was soft; there were positive McBurney and Rovsing signs. There was also tenderness and pain over the right lower quadrant. The white blood cells were 13,200, with a differential of 80 segmented leukocytes and 20 lymphocytes. Red blood cells were 4,600,000 and the hemoglobin 12.7 gm. per 100 ml. Appendectomy was performed the same day.

Gross Examination

The appendix measured 4 cm. in length, and its diameter was increased to 1.2 cm. The serosa was grayish yellow and was covered with fibrinopurulent exudate. The wall was friable. The lumen was dilated and filled with pus.

Microscopic Description

Microscopically, the appendiceal mucosa was extensively ulcerated and infiltrated with polymorphonuclear cells, lymphocytes, and eosinophils. Abundant balantidia were present in the base of the ulcers and in the wall. In the latter location they tended to be arranged in groups in the lymphoid follicles, where they had elicited a necrotizing reaction. They were found also in the lumina of lymph and blood vessels. One ulcer had perforated through the wall into the meso-appendiceal fat. The advancing border of this ulcer was represented by a group of

balantidia, some of which had degenerated. In general, the muscular part of the appendix was diffusely infiltrated with polymorphonuclear leukocytes, and edema was conspicuous everywhere.

The postoperative course was uneventful; unfortunately the feces were not examined, and it has been impossible to have the patient return for that purpose.

Case 5

A 37-year-old white woman (S.P. no. 75437) was admitted because of sharp pain in the right thigh, radiating to the abdomen. She felt perfectly well until the evening prior to admission, at which time the pain developed. It became worse on motion, which she was careful to avoid. She denied having had nausea, vomiting, diarrhea or urinary symptoms, or having had similar episodes in the past. She was in acute distress and the abdomen was distended. There was tenderness over the abdomen, which was accentuated in the right lower quadrant. There was rebound tenderness. A mass was felt in the right lower quadrant. Intestinal peristalsis was active. She was a gravida, XII, para XII, and had no history of any previous serious illness. The leukocyte count was 16,750, with a differential of 76 segmented leukocytes, 2 band cells, 16 lymphocytes, and 4 eosinophils. Urinalysis was negative. At laparotomy, both adnexae were inflamed. The appendix was removed. Grossly, its serosa was smooth and glistening; the serosal blood vessels were moderately congested and the distal end was slightly dilated. It measured 10 cm. in length and 1.2 cm. in diameter.

Microscopic Description

Microscopically, the serosa of the appendix revealed congestion and infiltration with eosinophils and polymorphonuclear cells. The muscle coat also was infiltrated with eosinophils and showed moderate interstitial edema. Sections of the distal end showed abundant balantidia and moderate numbers of leukocytes. There was no ulceration of the mucosa nor were any protozoa identified in the mucosa or other parts of the wall. There was no evidence of inflammation. The postoperative course was uneventful.

Discussion

The severity of the histopathologic changes found in our cases corroborates the opinion of those who believe firmly in the pathogenic nature of *B. coli*. They also illustrate several other factors which we deem advisable to discuss further. Bowman (*J. A. M. A.*, 1911) and later Jaffé (1919) stated that the living protozoon secretes a toxic substance, but in such small amounts that only mild or no inflammatory reaction is induced in the surrounding tissues. However, when they are very numerous or degenerating, a larger release of this toxin provokes an inflammatory infiltrate composed chiefly of lymphocytes and eosinophils. Harms corroborated these findings. The presence of polymorphonuclear cells is secondary to necrosis and possibly also to secondary bacterial infection.

It is obvious to us that the simple presence of balantidia is not enough to induce pathologic alterations. Other factors must act before this protozoon becomes pathogenic. In regard to dietetic habits, it has been repeatedly stressed that in most cases the patients lived on diets composed chiefly of carbohydrates with very little or no protein and scarce animal fats. Schumaker (1931) was able to correlate diet and intensity of infection in the hog as an experimental animal. However, despite the fact that poor eating habits affect large sectors of the population in many parts of the world, the number of cases of balantidiasis is indeed low.

Other circumstances must be present so that the combination of all will lead to a terrain favorable to the attack of balantidia. Masing was the first to point out the presence of achlorhydria or severe hypochlorhydria. Similar observations have been made by Brea and Nieto, Jarpa and Allende, Elliott and Hotson, Ferri, Cruz and Domingo, and Logan. This aspect of the problem has not been studied systematically. It is of interest that most cases, particularly the more severe ones, occur among elderly individuals, whose tendency to low free-acid values in the gastric contents is well known. In general, any condition that tends to depress the natural defenses of the body, such as chronic infection, undernourishment or starvation, alcoholism, and filth, seems to predispose man to infections by this organism. Furthermore, *B. coli* is not, in most patients, the only intestinal parasite present. Burrows and Jahnes found intestinal parasites of two to seven different species in their cases; likewise Bowman (*Philippine J. Sc.*, 1911), Santos, Masing, Kipschidse, Atiles, Sommerville, Koppisch and Wilking, Pramanik, and Stewart all observed concomitant parasitizations. The accompanying parasites were mostly *Ascaris lumbricoides, Necator americanus, Trichuris trichiura,* and *Strongyloides stercoralis,* but *Diphyllobothrium latum, Taenia saginata, Giardia lamblia, Blastocystis hominis,* and *Endamoeba histolytica* also have been reported. Atiles claimed that the degree of parasitization, state of nutrition of the host, nature of the host's diet, and quality of the intestinal flora all have an influence on the development of balantidiasis.

All of our cases came from a sector of the population in which both food habits and hygienic conditions were substandard. Undernourishment was evident in all, and two had severe chronic infectious diseases (tuberculosis and a typhoid carrier state). All cases which were necropsied showed multiple infestation by several kinds of intestinal parasites (Fig. 14). Unfortunately, no gastric analyses were done.

Our case of acute balantidial appendicitis is the third one recorded.

Campos' case was observed at necropsy as part of a diffuse inflammation of the whole colon and appendix, so that it does not count as surgical material. Our case 1 is interesting also because it was complicated by one of the less common manifestations of balantidiasis, namely, ulceration of the ileum with perforation. Only one case, that of Strong, has so far been reported in which a similar situation occurred.

The lesions of balantidiasis cannot be differentiated grossly from those of amebic dysentery and only histologic examination will reveal the true nature of the causal agent. However, because of the rapidity with which balantidia disintegrate, the organism may not be identified in cases in which the necropsy has been delayed for many hours, especially under the unfavorable circumstances of the tropics, unless a careful and painstaking search is carried out.

Summary

This study was based on 5 necropsies of patients dying with *Balantidium coli* dysentery and two surgically removed appendices, also infected by this protozoon. The lesions are similar, grossly, to those seen in amebic dysentery. The histopathologic differentiation is made by the identification of the ciliate, which usually is seen at the invading edge of the ulcers or at the periphery of submucosal abscesses. The lesions are seen also deep in the intestinal wall, commonly within lymphatic vessels. Occasionally also they are identified in the regional lymph nodes, where a mild reaction may occur. Only one case has been reported previously in which this parasite affected the terminal ileum; our case is the second one. Similarly, only two cases of acute balantidial appendicitis had been recorded, ours being the third. The literature in regard to geographic distribution of the parasite, incidence, epidemiology, biology of the ciliate, pathogenetic aspects, clinical picture, and recent therapeutic trends is reviewed.

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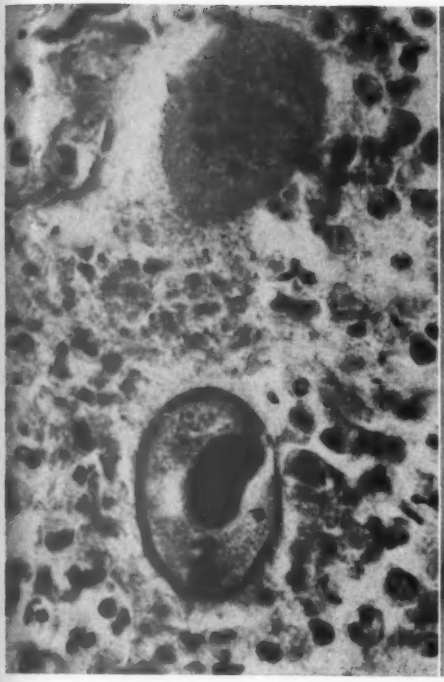
LEGENDS FOR FIGURES

FIG. 1. Trophozoite stage of *Balantidium coli*, degenerated above, well preserved below. Of note are the kidney-shaped nucleus, peristome, and cilia. Phosphotungstic acid-hematoxylin (PTAH) stain. × 800.

FIG. 2. Cystic stage, in feces. Delafield's hematoxylin stain. × 800.

FIG. 3. Immediately about the penetrating organism the cells may be shrunken, with acidophilic cytoplasm and pyknotic nuclei. Hematoxylin and eosin stain. × 360.



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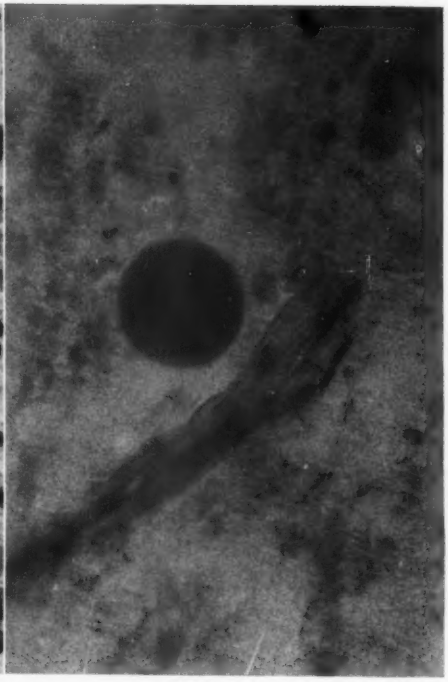

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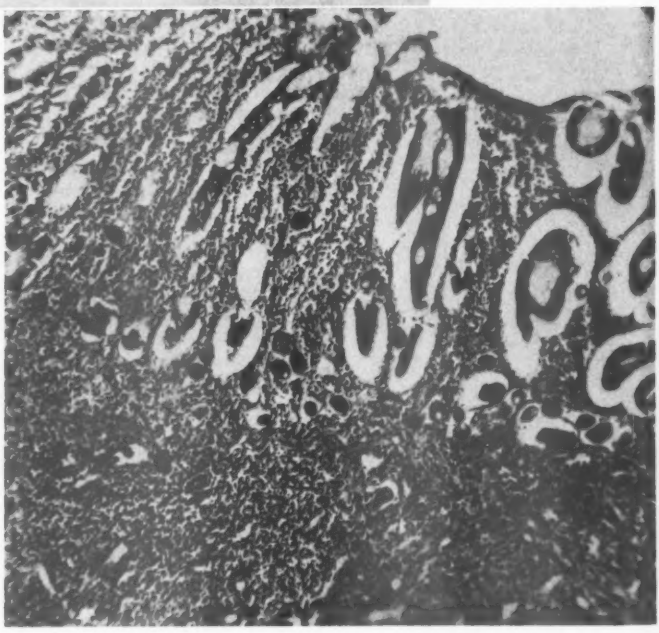

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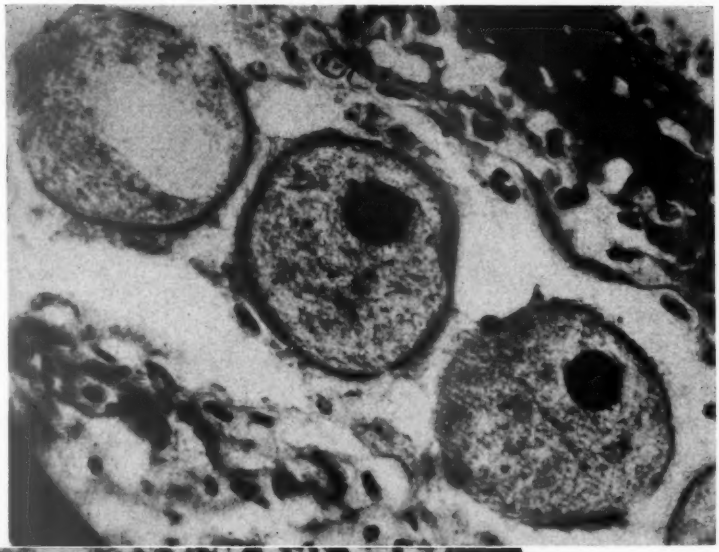
FIG. 4. Fatal case of balantidial dysentery. The ragged appearance of the mucosa is evident. There were multiple ulcers from the rectum to cecum. Perforation of the colon had occurred.

FIG. 5. Balantidial colitis: numerous organisms in tissues and in dilated lymphatics; marked lymphocytic and monocytic infiltration without ulceration. Hematoxylin and eosin stain. × 80.



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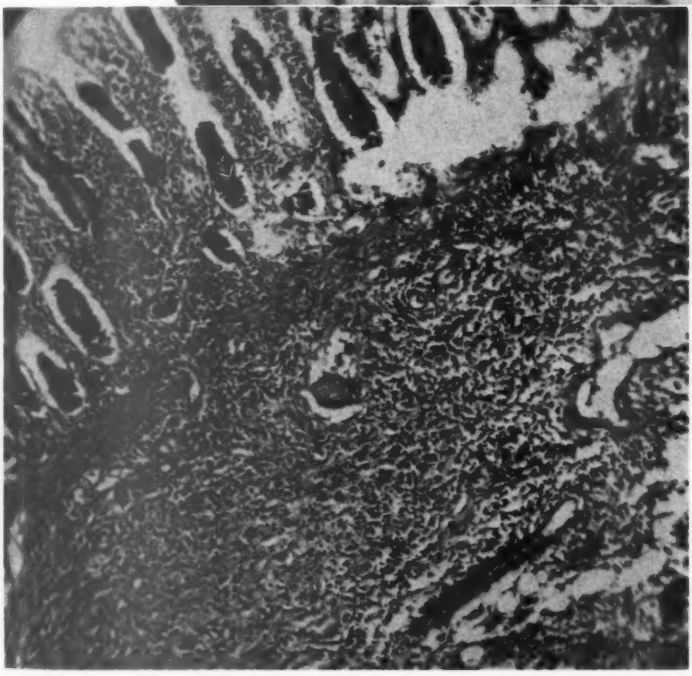

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FIG. 6. Balantidia in dilated lymphatic vessels of submucosa. PTAH stain. × 800.

FIG. 7. Zone of necrosis in submucosa surrounded by numerous round cells; in the center a degenerated balantidium may be noted. Hematoxylin and eosin stain. × 80.

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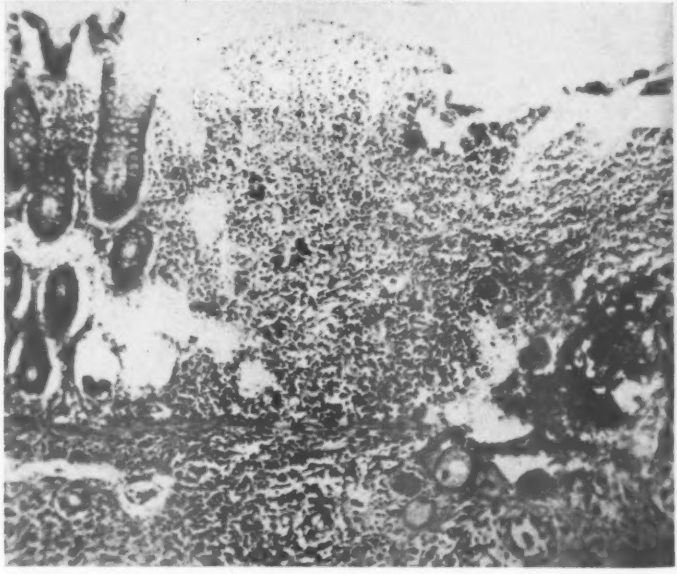

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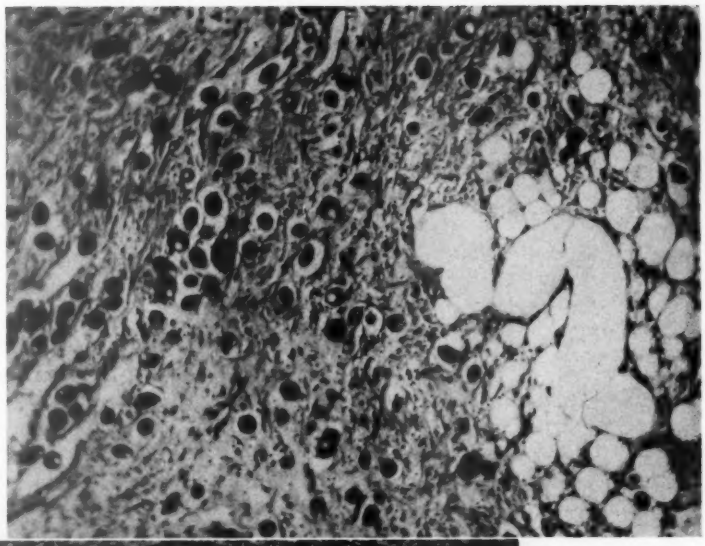
FIG. 8. Colitis: undermined margin of ulcer with necrotic base, below which there are numerous organisms. Hematoxylin and eosin stain. × 80.

FIG. 9. Necrotic material filling crater of ulcer; numerous balantidia, some perforating through the muscularis mucosae. Hematoxylin and eosin stain. × 80.



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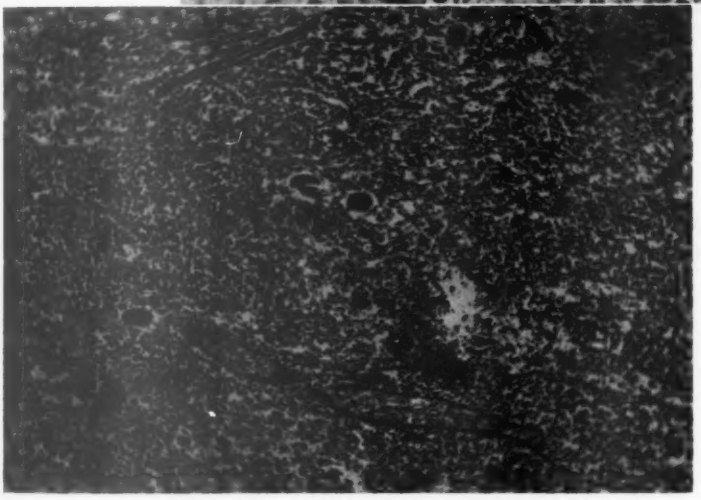

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FIG. 10. Colitis: numerous balantidia invading pericolic fatty tissue, possibly by agonal or post-mortem wandering. Hematoxylin and eosin stain. × 80.

FIG. 11. Lymph node from mesocolon with several balantidia; hemorrhagic and inflammatory reaction caused by the ciliate. Hematoxylin and eosin stain. × 80.

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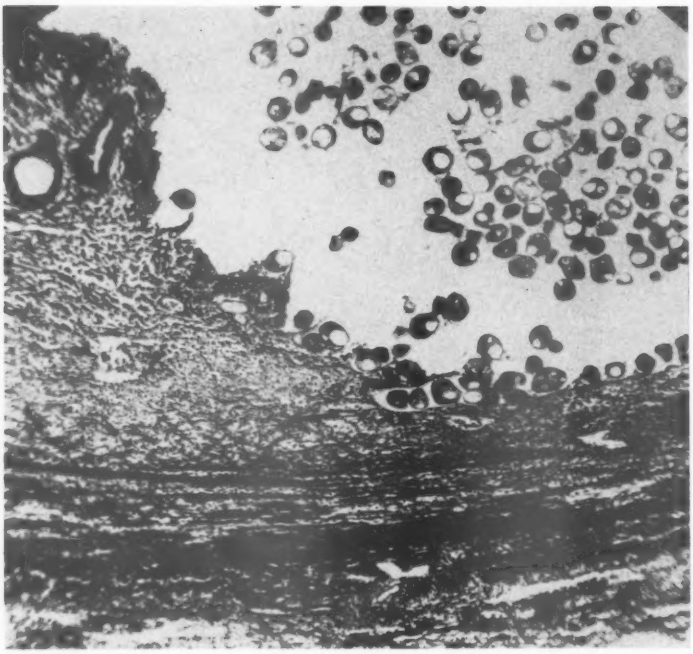
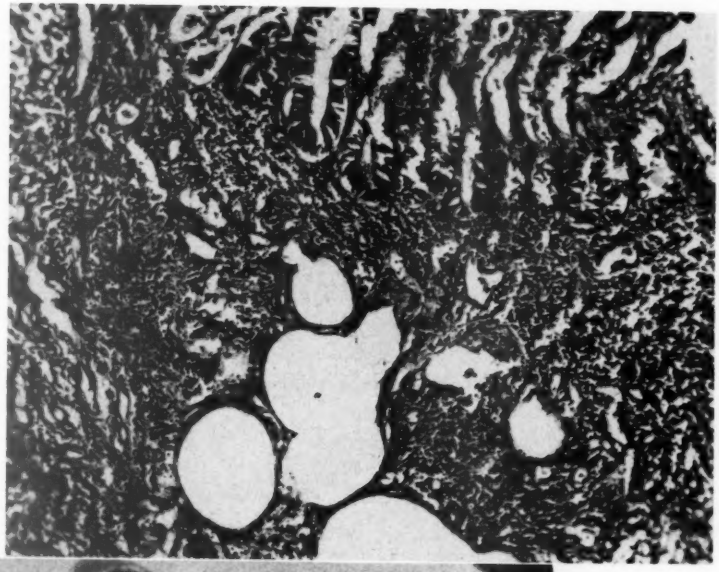


FIG. 12. Necrotizing balantidial appendicitis. Hematoxylin and eosin stain. × 80.

FIG. 13. Marked dilatation of mucosal lymphatics in a case of balantidial dysentery. Hematoxylin and eosin stain. × 80.

FIG. 14. Section from rectum showing implanted trichuris and two balantidia in lumen. Hematoxylin and eosin stain. × 80.



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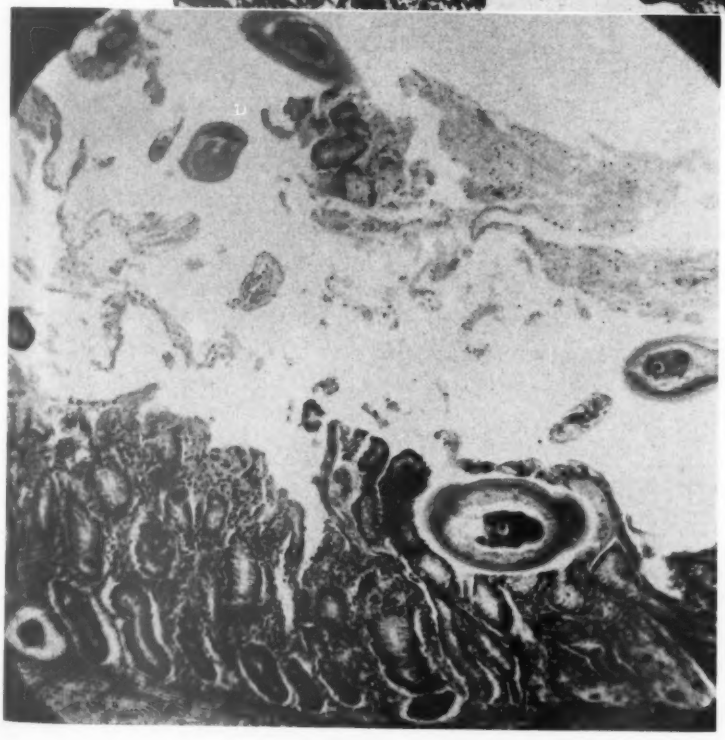

14

ELECTRON MICROSCOPIC OBSERVATIONS ON INTRACELLULAR RICKETTSIAE *

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The rickettsiae are recognized readily by ordinary microscopy in properly selected and prepared smears and sections because of their size and tinctorial properties. The identification by electron microscopy of individual organisms in purified suspensions is facilitated by their bacteria-like structure. Thus, rickettsiae-infected tissue seemed promising material for a comparative study by ordinary histologic methods and by electron microscopic examination of ultrathin sections. The present report is concerned for the most part with the cytopathology, as revealed by electron microscopic observations, of infection of the yolk sac tissue of the chick embryo by *Rickettsia mooseri.*

MATERIALS AND METHODS

Infected yolk sac tissue for examination was obtained in the following manner. Six-day-old chick embryos were inoculated into their yolk sacs[1] with about $10^3 LD_{50}$ of the egg-adapted line of the Wilmington strain of *R. mooseri.* After 4 to 8 days of inoculation at 35° C. the yolk sac tissues were harvested.

Specimens for electron microscopic study were fixed for 4 hours at 4° C. in 1 per cent osmic acid buffered at pH 7.4 with acetate-veronal.[2] The fixed tissue was dehydrated by placing it for intervals of 15 minutes in the following series of solutions: buffer, 25, 50, 70, 80, and 95 per cent ethanol and, finally, in three changes of absolute ethanol. The tissue was then immersed for 15 minutes in a mixture of equal parts of absolute ethanol and methacrylate monomer. The monomer consisted of one part methyl methacrylate and nine parts n-butyl methacrylate. Next, the tissue was placed for similar periods in two changes of undiluted monomer and, finally, put into monomer containing 1 per cent catalyst. The latter was an equal mixture of 2,4-dichlorobenzoyl peroxide and dibutyl phthalate. The individual specimens in the monomer-catalyst mixture were stored in gelatin capsules overnight at 47° C. to facilitate polymerization of the methacrylate. Sections approximately 0.05 μ in thickness were cut with a

* Received for publication, January 26, 1956.

Servall "Porter-Blum" microtome[3] and were examined with an RCA EMU 2C electron microscope.

Portions of each yolk sac were fixed also in Zenker's fluid, and paraffin sections stained by Giemsa's method were studied with the light microscope. Smears of each tissue specimen stained by Macchiavello's technique also were examined in order to estimate the number of rickettsiae present. If such smears contained only a few organisms, rickettsiae in ultrathin sections could be located only after considerable scanning with the electron microscope. In order to restrict the need for prolonged searching, only those specimens which showed numerous rickettsiae in their smears were examined intensively by histologic methods.

Suspensions of purified rickettsiae were prepared from infected yolk sacs according to the method of Wisseman *et al.*[4] Portions of these either were fixed for 30 minutes in 1 per cent osmic acid or were left untreated. Drops of fluid containing the osmium-fixed or unfixed suspensions were placed to dry on collodion-covered grids. These were shadowed subsequently with gold-manganin or chromium and examined with the electron microscope.

The transverse diameters of sectioned rickettsiae and of the rickettsiae in purified preparations were measured at 12 × magnification on the electron microscope plates by means of a comparator with a micrometer stage. The image magnifications were computed from measurements of standard latex particles photographed at each of the magnifications used in the study.

Results

Cytopathology of Infected Cells

Light Microscopy. Minimal pathologic changes were observed by light microscopy in stained paraffin sections of yolk sac tissue infected with *R. mooseri.* An inflammatory reaction such as would be accompanied by an influx of phagocytic cells was totally absent.* Because

* The absence of a phagocytic or other inflammatory response in the current specimens, despite the presence of phagocytes in the perivascular connective tissue of the normal yolk sac,[5,6] contrasts with histologic findings in other tissues infected with rickettsiae. For example, Hamilton,[7] in examining chorioallantoic membranes containing lesions caused by *Rickettsiae tsutsugamushi,* noted marked mesodermal proliferation and infiltration with ameboid "stem cells," some of which contained ingested rickettsiae. In addition, Pinkerton[8] observed an intense inflammatory reaction in scrotal tissues of guinea-pigs infected with *R. mooseri.* However, he encountered rickettsiae in the serosal cells of the tunica, and occasionally in endothelial cells, without finding them in mononuclear phagocytes or in connective tissue cells. It is of some interest in this connection that Castaneda's studies on opsonins[9] showed that rickettsiae were ingested *in vitro* by polymorphonuclear leukocytes but not by monocytes.

only scattered, single epithelial cells contained easily recognizable rickettsiae, one gained the impression that the number of infected cells was relatively low and that the total number of organisms was small. In such infected cells the rickettsiae were densely packed in large clusters; small groups of intracellular organisms were rarely observed. In contrast to the relatively few organisms visible in sections of infected yolk sacs, abundant rickettsiae were found in the smears stained by Macchiavello's technique from the same specimens. This disparity between the relative scarcity of organisms in sections of infected yolk sacs and their abundance in smears may have resulted from artifacts produced during preparation of paraffin sections. For example, the numerous cytoplasmic lipid granules normally present in epithelial cells were dissolved by organic solvents used for dehydration and clearing, thus leaving only thread-like septa as the sole remnants of their cytoplasm (Fig. 1). All but the large masses of rickettsiae may have been rendered unrecognizable by this distortion. Similar discrepancies had been noted by Wolbach *et al.*[10] in the numbers of rickettsiae observed in smears and sections of louse gut infected with *Rickettsia prowazekii*.

Electron Microscopy. In ultrathin sections of infected yolk sac tissue studied by electron microscopy, rickettsiae were encountered in mesothelial and epithelial cells (Figs. 2 to 7), but not in fibroblasts, macrophages, and cells of the vascular system. Infected epithelial cells were more numerous than infected mesothelial cells and, presumably by virtue of their greater size, generally contained more rickettsiae. However, the large lipid granules of the epithelial cells were singularly distorted during preparation of specimens for electron microscopy, and this interfered severely with satisfactory visualization. As this problem did not arise with the mesothelial cells, most of the observations and illustrations presented in this paper were obtained from study of the latter. This procedure was considered valid because no consistent differences in rickettsial structure or in cytopathologic changes were noted in infected cells of the two types.

The examination of numerous sections prepared at various stages of infection suggests the following developmental pattern for the cytopathologic lesion. In the earliest stages, a cell contains only a few rickettsiae in its cytoplasm and shows no discernible alteration of its normal constituents, i.e., nucleus, mitochondria, endoplasmic reticulum, and fine fibrillar component of the cytoplasm (Figs. 3 and 6). As the number of rickettsiae within the cell increases, there is a corresponding reduction in quantity of the submicroscopic cytoplasmic

organelles, notably the endoplasmic reticulum and the fine fibrils (Figs. 4 and 7). In the terminal stages of infection the cell becomes essentially a sack filled with rickettsiae (Fig. 5). At this point most of the normal intracellular structures have disappeared leaving a pyknotic nucleus and a few mitochondria. Although reduced in number, the remaining mitochondria of such cells give no morphologic indication of being damaged (Fig. 6). Heavily infected cells eventually rupture, releasing their content of rickettsiae. When this stage is reached the disruption of the histologic architecture of the yolk sac in such necrotic areas impedes further study by electron microscopy.

Rickettsial Morphology

In one of the earliest electron microscopic studies of rickettsiae, Plotz *et al.*[11] examined partially purified suspensions of formalinized rickettsiae prepared from infected yolk sacs. The basic structure of the organisms was the same in each species studied; a limiting membrane surrounded an inner protoplasmic core in which numerous dense granules were imbedded. Subsequently, Ris and Fox[12] demonstrated in Feulgen-stained smears of concentrated suspensions of *R. prowazekii* that the granules within the protoplasmic core contained desoxyribonucleic acid.

In the present study the electron microscopic appearance of intracellular rickettsiae in sections of infected yolk sac tissue resembled that of organisms in purified suspensions. When observed *in situ* within the cytoplasm of a cell, the rickettsiae were extremely dense, i.e., they scattered a high proportion of the incident electrons. Nevertheless, small areas of even greater density were discernible throughout the protoplasm of thinly sectioned rickettsiae (Fig. 6). The granular structures in the protoplasm were poorly visualized in more opaque rickettsiae encountered in slightly thicker sections (Fig. 7). Electron micrographs of shadowed, unfixed washed rickettsiae showed little evidence of diffuse granulation (Fig. 8). The protoplasm was concentrated in a thick, central mass giving the organism the appearance of a fried egg. This concentration of material could have occurred during the preparation of the purified suspensions of rickettsiae or during their subsequent processing for electron microscopy. To determine when this change took place, purified suspensions of rickettsiae were treated with osmic acid so that the organisms were fixed while still in aqueous suspension. The electron microscopic appearance of these fixed rickettsiae is illustrated in Figure 9. The protoplasm, which extended practically to the outer limit of the organism, con-

tained numerous spheroidal granules. It seems likely, therefore, that the unfixed rickettsiae acquired their "fried egg" appearance while being dried and shadowed prior to examination with the electron microscope.

Rickettsiae in ultrathin sections and washed organisms in suspension exhibited differences in the structure of their external limiting membranes. The protoplasmic cores of *R. mooseri** in infected yolk sac tissue were rarely encased by continuous membranes, although such structures were encountered occasionally (Fig. 7). In contrast, the cores of washed rickettsiae were surrounded invariably by a rim of relatively homogeneous material. This rim was present in both osmium-fixed and unfixed rickettsiae, but was narrower in the former.

TABLE I

Comparison of the Width of Intracellular and Washed Rickettsiae

Preparation	No. of rickettsiae examined	Minimum and maximum width	Average width
		mμ	mμ
Intracellular rickettsiae fixed *in situ*			
(a) Rickettsiae in the same sections and specimen block used for Figs. 6 and 7	10	225–375	287
(b) Rickettsiae in the same yolk sac as (a) but from a different specimen block	10	188–282	246
(c) Rickettsiae in a different yolk sac	20	166–281	202
Rickettsiae in suspensions			
(d) Unfixed, washed rickettsiae similar to those shown in Fig. 8	20	376–584	489
(e) Fixed, washed rickettsiae similar to those shown in Fig. 9	20	446–620	508

Preliminary observations suggested that rickettsiae in sections were not as wide as those in purified suspensions of washed organisms. Accordingly, careful measurements were made of individual organisms from different preparations. Only the widths were measured because rickettsial pleomorphism seriously hampered a comparison of lengths and areas. The average values recorded in Table I for three preparations of intracellular organisms were different, but the range of measurements overlapped and the median value was about 250 mμ. In contrast, the organisms in purified suspensions measured about 500

* In this laboratory, membranous structures enclosing *R. tsutsugamushi* have been observed consistently with the electron microscope in sections of infected chorioallantoic membranes and in sections of roller tube cultures of infected MB III cells (mouse lymphoblasts).

mμ in diameter. Several factors could contribute to the difference in size under the two types of conditions. First, the true diameter of a sectioned rickettsiae would be observed only if the plane of section coincided with, or crossed at right angles to, the longitudinal axis of the organism. Chance sectioning at such planes would be uncommon and variations in average diameters of groups of organisms, such as mentioned under (a), (b), and (c) in Table I, might be anticipated. It may be noted that the differences in values (a) and (b) are not statistically significant while those between (a) and (c) and between (b) and (c) are significant. Second, flattening of washed rickettsiae by the force of their own weight and by the action of surface effects during drying on the collodion-covered grids undoubtedly increased their widths. Third, also with regard to the washed rickettsiae, the technique of differential centrifugation employed in the purification procedure selected particles of uniform density. Moreover, the procedure was designed to eliminate from the final suspension nearly all of the soluble materials and the cellular organelles present in the original homogenates. If a considerable proportion of the smaller rickettsiae were discarded during the procedure, a preponderance of large organisms would be found in the final, purified preparation. The effect of such factors as the aforementioned must be evaluated before the observed differences in width of rickettsiae under the present conditions can be accepted as important.

The present study has added little to our knowledge concerning the method of multiplication of this pleomorphic, coccobacillary organism. The appearance of diplobacillary forms in sections of infected yolk sac (Figs. 3 and 6) suggested a process of division, but it would be fruitless at this stage to go beyond the speculations of others who have dealt with the multiplication of rickettsiae on the basis of findings by light microscopy. Wolbach's comments about *Rickettsia rickettsii*[13] epitomize these opinions: "Appearances are found which would indicate that these rods divide by transverse fission, and that the division is preceded by a division of the chromatoid particles, but such evidence is purely morphological."

Discussion

R. mooseri in osmium-fixed, ultrathin sections of yolk sac tissue was readily distinguished from normal intracellular structures during electron microscopic examination because of its greater opacity, its circular or rod-like silhouette, and its visible internal granulation. The morphologic appearance of rickettsiae in such sections was consistent

with that described in this and previous electron microscopic studies of purified rickettsial suspensions.[4,11] The rare presence of an external limiting membrane associated with organisms sectioned *in situ*, while difficult to explain in view of its consistent demonstration in washed organisms isolated from the same tissue, was not sufficient to interfere with their identification. The sole cytoplasmic structures with which intracellular rickettsiae might have been confused were mitochondria, because in size and outline they bore some resemblance to one another. In sections both were either circular or rod-like in outline, although the mitochondria generally were broader. Their internal structures, however, were strikingly different and prevented any confusion of the two entities. The mitochondria were surrounded by a double membrane, the inner half of which was invaginated in centripetal folds or cristae. Between the cristae, the ground substance of each mitochondrion was of the same order of density as that of the cytoplasmic matrix. In contrast, rickettsiae in sections rarely exhibited a limiting membrane, and their cores were markedly denser throughout than was the cytoplasmic ground substance.

The explanation for the inconstant appearance of the external limiting membrane surrounding rickettsiae observed *in situ* is at present unknown. Recent morphologic studies show that viruses differ one from another in the occurrence of surrounding membranes. Morgan *et al.*[14] reported that intranuclear particles of herpes simplex virus growing in the chick chorioallantois are surrounded by a single membrane, whereas intracytoplasmic and extracellular particles possess a double membrane. In a study of vaccinia and fowl pox viruses,[15] this same group has shown that the intracytoplasmic virus particles usually are surrounded by a single membrane whereas extracellular particles possess a second membrane. The authors suggest that the acquisition of the additional membrane makes the structure into a mature virus particle. A more complicated picture of membrane formation has been described by Reissig and Melnick,[16] who studied the growth of a member of the herpes virus group in infected tissue culture cells and observed virus particles with one or two surrounding membranes in both the nucleus and the cytoplasm. These observations suggest that there may be no universal pattern of membrane development which characterizes viruses developing in a nuclear or cytoplasmic environment.

The present study illustrates the diverse occurrence of the limiting membrane of rickettsiae under different conditions. *R. mooseri* observed *in situ* in infected yolk sacs usually lacks a limiting membrane while washed organisms obtained from the same kind of tissue always

possess such a structure. Furthermore, *R. tsutsugamushi* observed in sections of infected chorioallantoic membranes and of infected tissue culture cells invaribly possesses a discernible limiting membrane. Any discussion of the limiting membrane of rickettsiae should mention the concept of the capsular substance which Shepard and Wyckoff[17] described in their report of electron microscopic studies of *R. mooseri* and *R. prowazekii*. Extraction with ethyl ether alters the surface of rickettsiae and liberates soluble rickettsial antigen.[17] If rickettsiae are indeed enveloped by a capsular substance as well as by a limiting membrane, certain of the variables encountered in the current work might be accounted for by differences in composition of the envelope under different circumstances.

In osmium-fixed, washed rickettsiae, the granules appear as small, scattered spheroids imbedded in the matrix of the core (Fig. 9). The granules can be distinguished from the intergranular substance because the latter, which seems to be less resistant to forces acting during drying and shadowing even after fixation, collapses around them and allows their contours to project from the surface of the organism. A slightly mottled appearance of the protoplasmic core in sectioned rickettsiae is the sole indication of their presence (Fig. 6). If one assumes from the study of purified suspensions that granules exist in the living organism and that their origin is not artifactitious, one might explain the failure to see them more clearly in sections on the basis of a lack of differential "staining" of the intergranular and granular substance by osmium, or one might postulate a redistribution of components within the rickettsiae during fixation which obscures or destroys the structure of the granules. Altered viral structure arising from a redistribution of protein material has been noted in washed elementary bodies of vaccinia.[18] Here the granules normally present within Paschen bodies were more clearly seen with the electron microscope after nucleoprotein was extracted with dilute alkali.

Summary

A comparative study of chick embryo yolk sac infected with *Rickettsia mooseri* was made by ordinary histologic methods and by electron microscopic examination of ultrathin sections. Rickettsiae were readily differentiated from normal intracellular constituents of infected cells during electron microscopic examination because of their characteristic structure.

Rickettsiae *in situ* were found only within the cytoplasm of mesothelial and epithelial cells of the yolk sac where they appeared to cause

lysis of the submicroscopic cytoplasmic components. In the infected yolk sacs, tissue reaction in the form of an inflammatory exudate was not observed.

R. mooseri observed with the electron microscope in ultrathin sections of infected yolk sacs appeared as dense, spherical or rod-shaped bodies approximately 250 mμ in width and up to 1.2 μ in length.

Shadowed, washed *R. mooseri* obtained from infected yolk sacs and examined with the electron microscope had a granular core of protoplasm enveloped by an external limiting membrane. The protoplasm appeared less distorted when the rickettsiae were fixed in suspension with osmic acid prior to shadowing.

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LEGENDS FOR FIGURES

The photomicrograph and all of the electron micrographs which illustrate sections of infected yolk sacs were made from membranes removed from 12-day-old chick embryos that had been infected with *Rickettsia mooseri* 6 days prior to fixation of the specimens.

FIG. 1. Photomicrograph of a region of infected yolk sac. The mesothelial cell layer composed of cells resembling squamous epithelium covers the upper surface of the membrane. A capillary containing blood cells lies in the connective tissue beneath the mesothelium. The tall columnar cells with their bases resting on the submesothelial connective tissue comprise the epithelial cell layer. The apices of the epithelial cells normally border the yolk fluid, but in this specimen only scattered clumps of yolk granules remain of this material. The cytoplasm of the epithelial cells is empty except for thread-like remnants of protoplasm. An epithelial cell near the center of the field contains clusters of small, dark bodies of spherical or rod-like shape which are identified as rickettsiae. The section was stained by Giemsa's method. The rickettsiae exhibit deep blue basophilia which contrasts sharply with the pink acidophilia of cytoplasmic remnants in the epithelial cells. × 1,840.

FIG. 2. An electron micrograph of a region of infected mesothelium at relatively low magnification. The mesothelium is a layer of imbricated, elongated cells in the upper portion of the picture. Beneath it lies the submesothelial connective tissue in which fibroblasts and barely visible collagen fibrillae are intermingled. The mesothelial cell in the center of the picture contains a number of rickettsiae which appear as small, dark bodies intermingled with normal mitochondria. Most of the organisms are grouped to the left of the nucleus. The adjoining mesothelial cells and the subjacent fibroblasts are free of organisms. At this magnification degenerative changes are difficult to recognize, but careful examination of the cytoplasm of the infected cell reveals diminution of submicroscopic cytoplasmic organelles in the region of the rickettsiae. × 5,720.

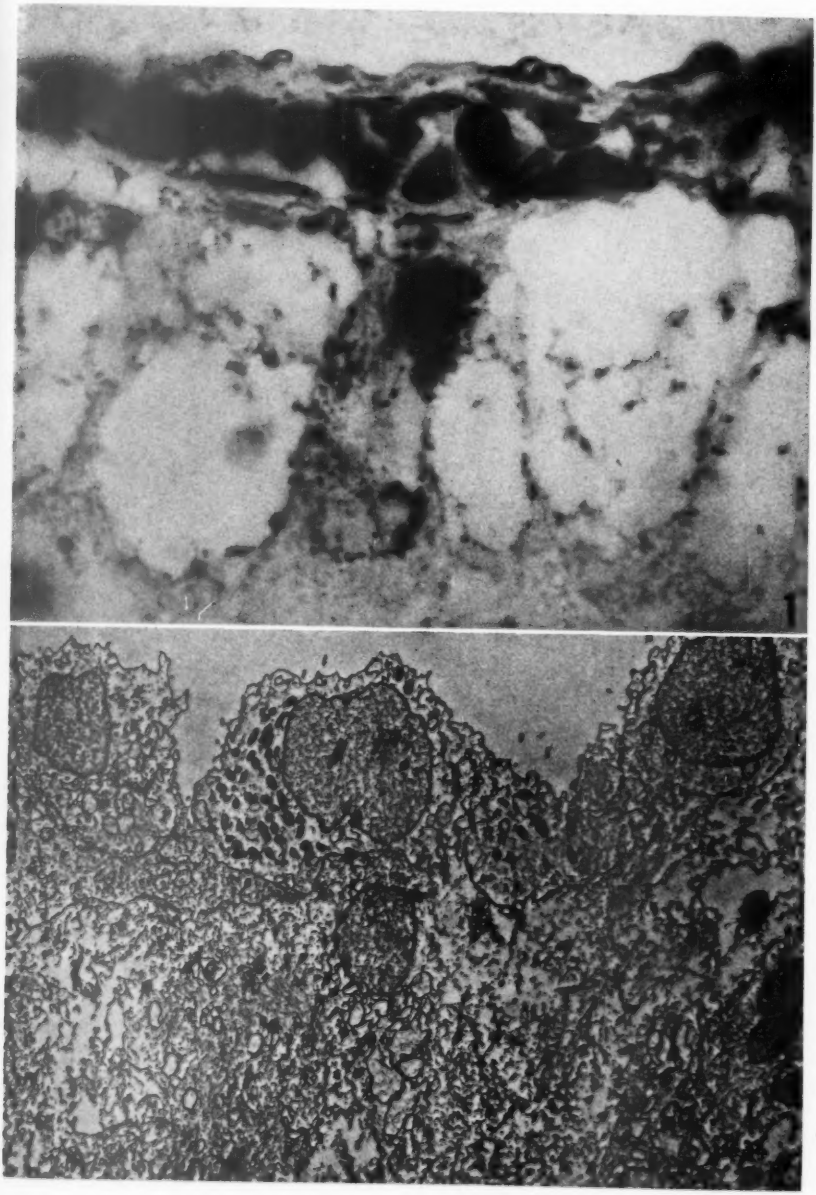


FIG. 3. An electron micrograph of an early stage of infection in a mesothelial cell. A single mesothelial cell, with its nucleus in the lower right corner of the picture and its cytoplasm extending in zigzag fashion to the upper left corner, occupies most of the field. Scattered collagen fibrillae of the submesothelial connective tissue lie below the cell. Above the nucleus, spherical and rod-shaped rickettsiae are intermingled with the normal organelles in the cytoplasm of the mesothelial cell. There is, as yet, no visible evidence of the destructive effect of the rickettsial infection, since the cytoplasm of the infected cell appears normal except for the presence of the organisms. × 21,000.

FIG. 4. An electron micrograph of an epithelial cell in an intermediate stage of infection. The cell membrane of the epithelial cell extends across the field from left to right, and the nucleus fills the lower left corner of the picture. Because of the close apposition of the basement membrane and the cell membrane, the latter appears double in certain regions. Above the epithelial cell, collagen fibrillae of the submesothelial connective tissue can be identified. Rickettsiae are scattered in the cytoplasm between the nucleus and the cell membrane of the epithelial cell. Many of the submicroscopic cytoplasmic organelles have disappeared from regions adjacent to the rickettsiae, but the mitochondria appear unaffected. × 14,700.

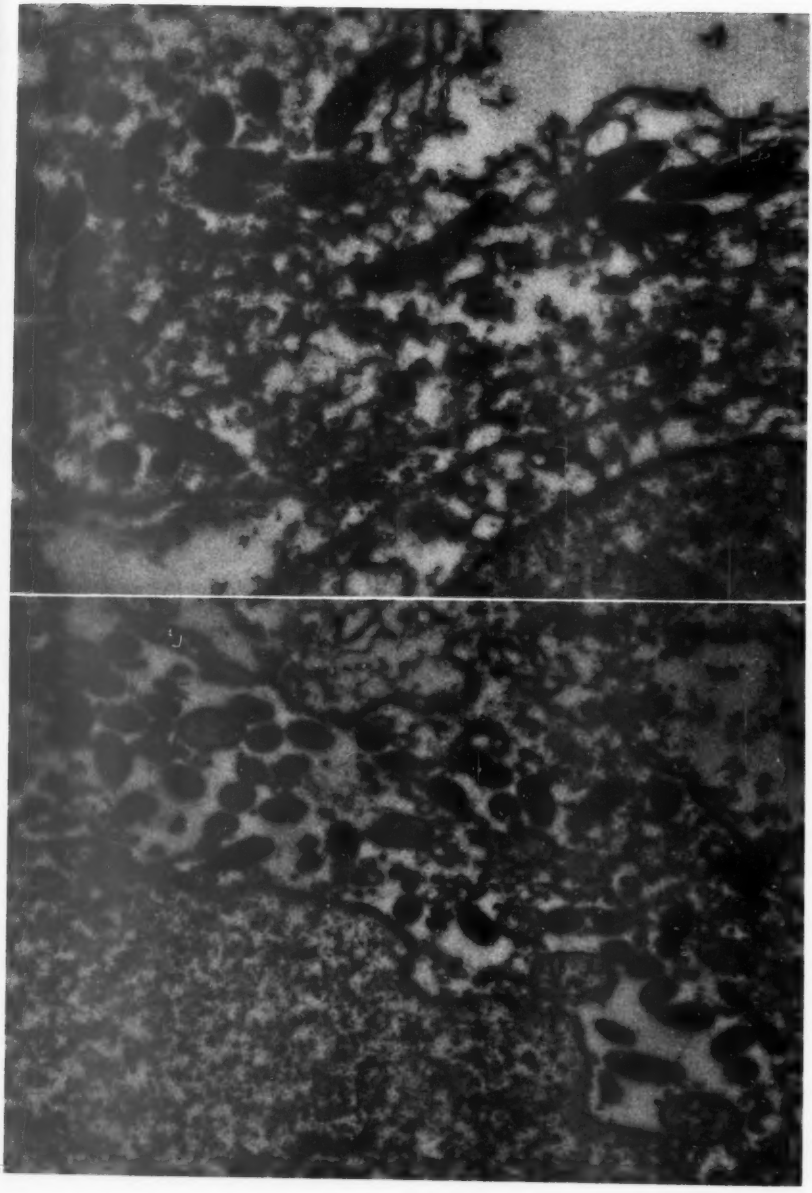
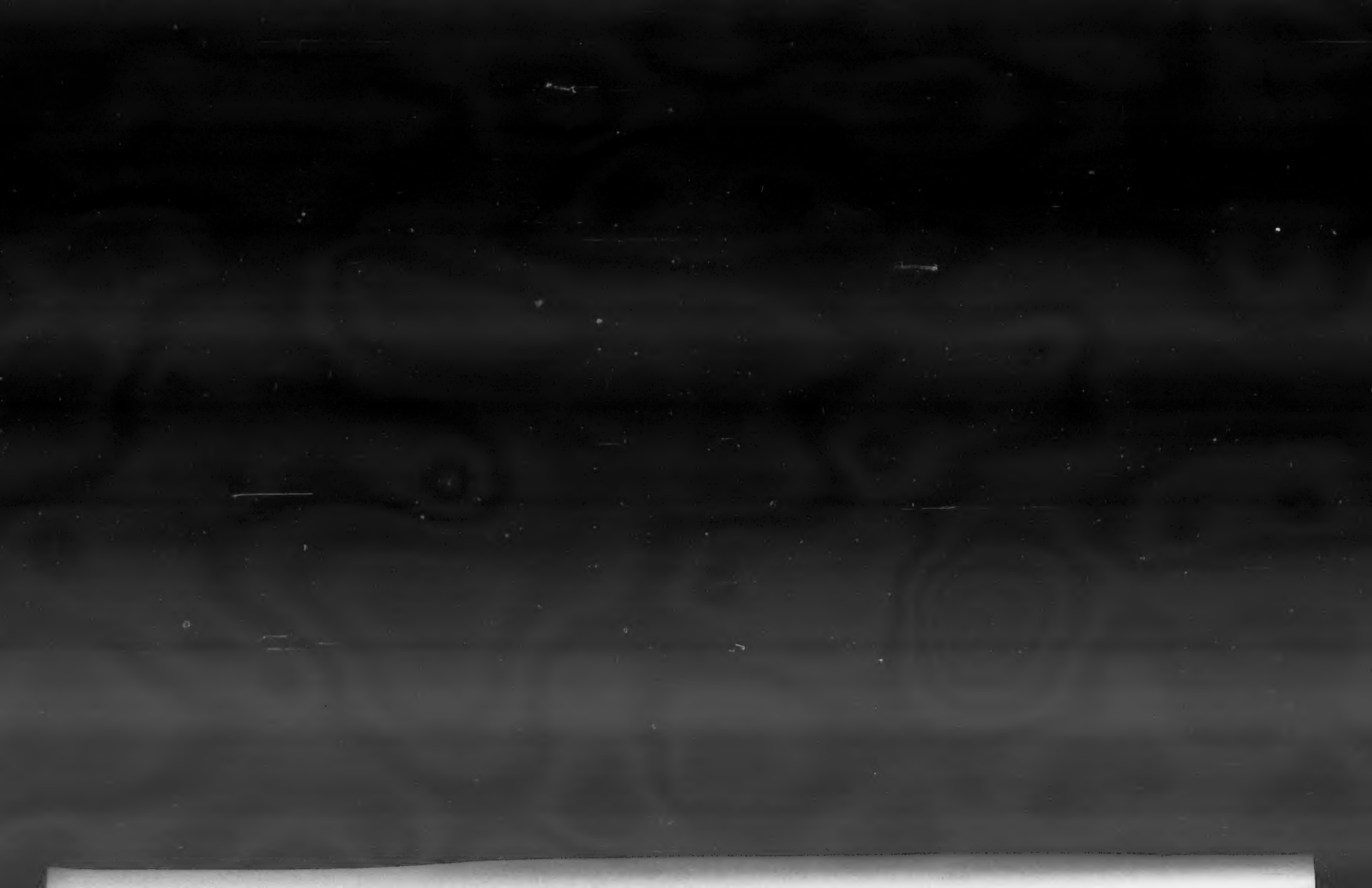


FIG. 5. An electron micrograph of a mesothelial cell in the terminal stages of infection. Scattered throughout the mass of rickettsiae in the cytoplasm are occasional mitochondria and granules. The fine fibrils and vesicular strands of endoplasmic reticulum normally present have disappeared. The pyknotic nucleus lying in the center of the cell contains scattered, dense clumps of chromatin. × 9,370.

FIG. 6. An electron micrograph of a portion of an infected mesothelial cell. Scattered rickettsiae are seen in an otherwise normal cytoplasmic background. Dark, granular structures are visible within the dense rickettsiae. The segments of the diplobacillary form in the lower right corner appear separated by a less dense, constricted band. × 53,300.



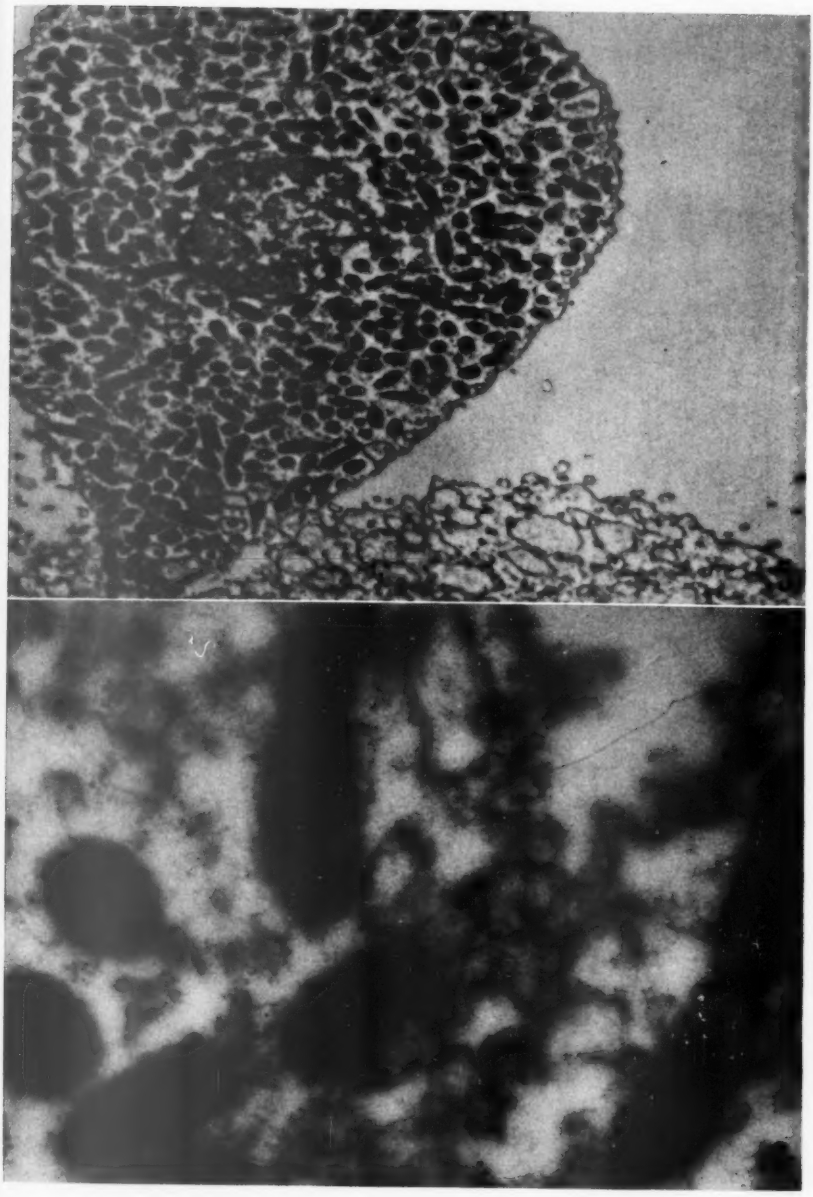
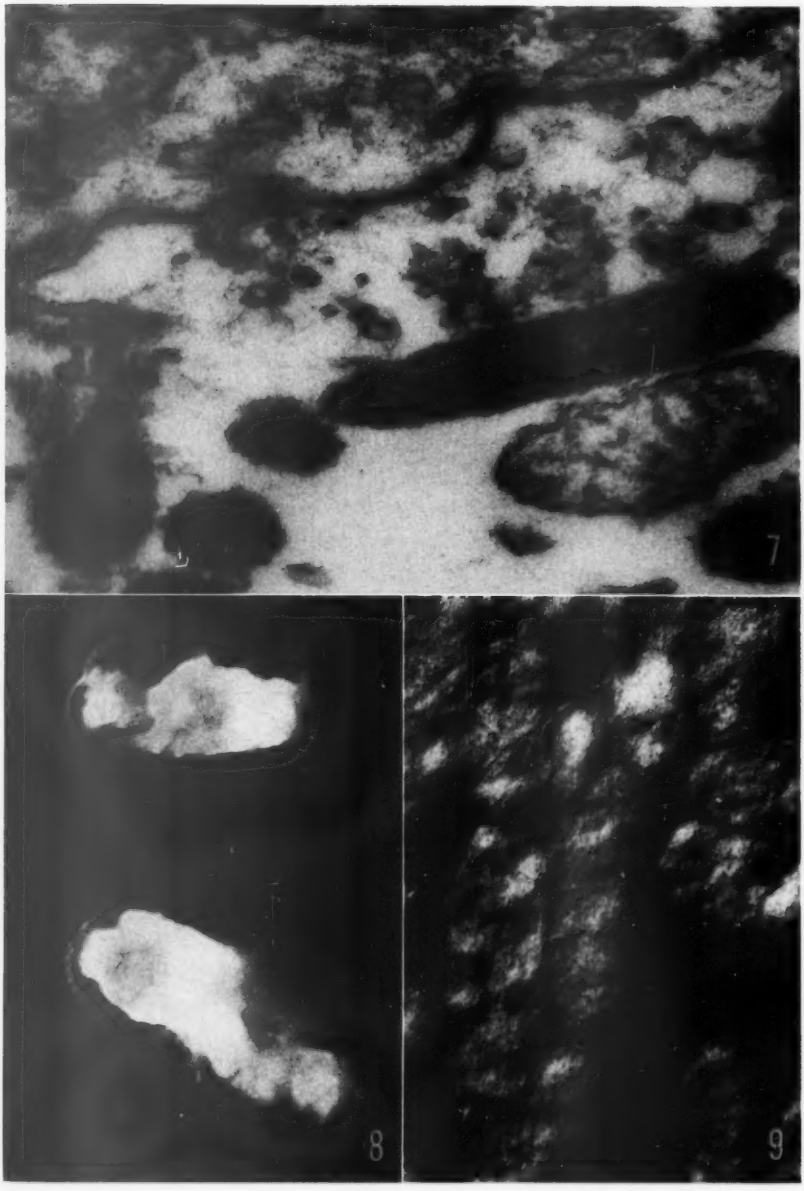


FIG. 7. An electron micrograph of portions of two adjacent mesothelial cells. The closely apposed cell membranes of the mesothelial cells run upward and to the right. The cytoplasm of the lower cell contains a number of dense rickettsiae and is essentially devoid of organelles except for a normal appearing mitochondrion and a few vesicles of endoplasmic reticulum. A scalloped, external limiting membrane can be discerned around nearly all of the organisms, but granules in their protoplasmic cores are not visible. × 53,300.

FIG. 8. An electron micrograph of a suspension of unfixed *R. mooseri* shadowed with chromium. These rickettsiae illustrate the "fried egg" appearance typical of such preparations. The protoplasmic core of each organism is concentrated in a central mass surrounded by a broad, shallow rim of material representing the external limiting membrane. × 52,800.

FIG. 9. An electron micrograph of a suspension of *R. mooseri* fixed with osmic acid while still in suspension and subsequently shadowed with gold-manganin. The appearance of these organisms resembles more closely that seen in sections of infected yolk sacs than that illustrated in Figure 8. The low spheroidal hummocks within their protoplasmic cores presumably represent granular masses. The external limiting membranes appear as narrow rims surrounding the cores. × 32,200.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EXPERIMENTAL OCULAR TRICHOMONIASIS

Pathologic Observations *

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The initial transmission of *Trichomonas vaginalis* to the eye of the rabbit with subsequent serial transfer through 14 successive passages has been reported.[1] The purpose of this paper is to describe pathologic observations on the eyes of the animals sacrificed at various times after injection of *T. vaginalis*.

Studies on *T. vaginalis* (Donné,[2] 1836) have been limited by the inability to transmit the parasite to animals. Trussell[3] listed numerous unsuccessful experiments on dogs, cats, mice, rats, rabbits, guinea-pigs, and many other species. Monkeys harbor a parasite which may be identical to *T. vaginalis*. Until recently the only successful animal transmission of the human parasite was to the vaginal tract of rhesus monkeys.[4] The difficulty of working with these animals and the inability to obtain bacteria-free trichomonas infections in the vaginal tract either of the human or monkey host handicapped efforts to evaluate systemic therapy.

Most of the efforts to transmit *T. vaginalis* to animals have been directed to implanting the parasites into the vaginal tract. Because of the failure of these efforts, other routes of infection have been attempted. Schnitzer, Kelly, and Leiwant[5] produced local infections in mice by intra-abdominal, intramuscular, and subcutaneous injections. Similar types of infection also have been produced by Kupferberg and his associates.[6] Inoki and Hamada[7] regularly caused death in young mice by pretreatment with intravenous injections of washed suspensions of erythrocytes of young chickens followed by intraperitoneal injections with *T. vaginalis*. Kelly and Schnitzer[8] demonstrated the development of immunity to re-infection in animals which previously had been infected experimentally. Recently Uhlenhuth and Schoenherr[9] reported that they were able to infect 20 to 30 per cent of hamsters intravaginally with *T. vaginalis*.

Previous studies indicated that *T. vaginalis* was invariably present in the urethrae of women who harbored the parasite in the vagina.[10]

* Received for publication, March 10, 1956.

Our preliminary attempts to produce *T. vaginalis* infections in the lower urinary tract of animals were unsuccessful and were not reported. On the theory that human parasites might behave like tumor cells we followed the work of Greene,[11] who utilized the anterior chamber of the eye of the rabbit for implantation of human tumor and embryonic tissue. Lwoff and Nicolau[12] injected *Trichomonas foetus* into the anterior chamber of the eye of a rabbit, causing severe lesions of the eyeball with death on the 35th day. The parasite was recovered from the brain.

Materials and Methods

The Organism and the Media Employed. The organism was a strain of *T. vaginalis* isolated on January 17, 1955, from a patient with vaginal and urethral trichomoniasis. The original isolation was accomplished on a trypticase medium[13] to which had been added penicillin and streptomycin.[14] The culture was transferred nine times at intervals of 1 to 4 days. It was then seeded into a thioglycollate medium to which had been added horse serum and one loopful of dry rice starch (THS medium).[1,13] A 2-day pure culture of this THS medium was the material utilized for injection in the eyes of 2 rabbits. All other rabbits were injected with aliquots of the aqueous humor of infected eyes. For dilution of the aqueous humor, when necessary, thioglycollate medium with serum, but without starch, was utilized (TH).

Method of Injection. Before making an injection into the anterior chamber, the eye was gently pushed out of its socket by pressing upward against the lower lid through the skin, and held there. Then 0.05 cc. of infective fluid was injected just inside the anterior chamber. Before withdrawal of the needle from the anterior chamber the eye was released and returned to its normal position. If this procedure was not carried out, seepage of the fluid occurred from the puncture point of the needle. The same procedure was used when aqueous humor was aspirated from the anterior chamber. Throughout, 26 gauge needles were used. It is probable that at times the lens was inadvertently injured, as will be described later. All manipulations of the eyes and inoculations and withdrawals of fluids were done under ether anesthesia.

Number of Eyes Injected. A total of 63 eyes were used in the primary experiment (Table I). Of these, 58 received injections into the anterior chamber and 5 into the vitreous body. Seventeen eyes were excluded from this series, leaving a total of 46, the study of which

forms the substance of this report. Eight eyes were eliminated because of bacterial contamination, 3 because no sections were made, and 6 for other reasons. The comparatively large number of contaminated eyes was due to the numerous punctures through the unsterilized cornea, and to the fact that aqueous humor had to be inoculated immediately into the next rabbit before tests for bacterial sterility could be completed.

To study the progression of the infection in the eye, 2 animals were sacrificed on the 1st day, 2 on the 2nd, 1 on the 3rd, 4 from the 5th to 10th day, 5 from the 11th to 20th day, and 13 from the 21st to 41st day. All rabbits were killed by the injection of air into the ear veins.

TABLE I

Summary of Experiments

Total number of eyes	63
Injected into anterior chamber	58
Injected into vitreous body	5
Number of eyes excluded	17
Eyes studied	46

Preparation of Eyes for Sectioning. The eyes were prepared for microscopic study as follows: All eyes were enucleated and fixed in 10 per cent formalin for several days. As a rule they were cut by midline horizontal total section. Preliminary staining included hematoxylin and eosin, Giemsa's, Papanicolaou's, Wright's, Tomlinson and Grocott's,[15] phosphotungstic acid-hematoxylin, Mallory's trichome, van Gieson's, Schiff's periodic acid, Weigert's, and several other techniques. Wright's and Tomlinson-Grocott's stains gave the best results.

Aqueous Humors. While the experiment was in progress and before sacrifice of the animals, numerous punctures of the anterior chambers were made. Some of the fluids were used for infecting the eyes of normal rabbits; others merely to test for the presence of the parasite. In all, 152 fluids were examined, one to eight aspirations being made in each eye. Both aerobic and anaerobic bacteriologic tests were carried out routinely on each aqueous humor transfer to determine whether a pure culture of the parasite was present. All of the fluids were examined for *T. vaginalis* by wet smear, by culture on THS medium, or by both methods, and many were examined also by dry smear with Wright's stain.

PATHOLOGIC OBSERVATIONS

Gross Examination

In the eyes which became infected, the cornea appeared slightly cloudy or red, or both, 2 to 4 days following injection. By the tenth day the cornea was definitely cloudy and sometimes there was a small opaque area at the site of injection. Through the pupil a yellow disk-

shaped or elliptical structure, presumably the lens, could often be seen well back of the cornea. In some animals which were sacrificed long after injection (33 to 41 days) the cornea was somewhat opaque; the contents of the anterior chamber could not be seen but a yellow disk-shaped body, probably the lens, occasionally could be recognized through the pupil.

Histologic Examination

The Cornea. In the 41 eyes injected into the anterior chamber with *T. vaginalis,* there were no specific alterations in the cornea. In several eyes the sites of perforation presented an acute, subacute, or chronic inflammatory reaction, depending upon the time of sacrifice of the animal. No invasion of the cornea by the parasite was found.

The Anterior Chamber. Except in three instances there was absence of parasites in sections of the anterior chamber despite the fact that the aspirates from this portion of the eye frequently were positive for *T. vaginalis.* Many additional sections were made in order to be sure that the parasites were not present in a portion of the anterior chamber not originally sectioned. The recuts were negative. The probable explanation was that most of the contents of the anterior chamber were lost in the preparation of the sections by the technique already described. This was established in supplementary experiments in which a pure culture of *T. vaginalis* was injected into the anterior chambers of 6 rabbits (12 eyes) which were sacrificed at once. Sections of these eyes prepared in the usual fashion were negative for parasites. In current studies efforts are being made to preserve the contents of the aqueous humor.

The Iris and Ciliary Body. No dramatic or specific alterations in the iris or the ciliary body were demonstrated in any of the sections. Congestion and a non-specific inflammatory reaction frequently were observed. Anterior synechia was common, being noted in 16 eyes. In view of the frequency of puncture of the anterior chamber, synechia was interpreted as a non-specific effect. In no sections did the parasite itself appear responsible for synechia.

The Posterior Chamber. Little of interest was found in the posterior chambers. In 6 eyes parasites were seen (Table II), but these were in such intimate contact with fragmented lens substance that it was difficult to determine if the parasites were actually within the posterior chamber or were simply associated with lens substance displaced in the preparation of the sections.

The Lens. The growth of *T. vaginalis* in the lens of the eyes of the rabbit was the most interesting finding. Of the 38 eyes from rabbits

killed 5 to 41 days after injection into the anterior chamber of *T. vaginalis*, 21 lenses were heavily infected and one lens was slightly infected with the parasite. In 19 of these 22 eyes the aqueous humor had been positive at least once and of these, 12 had been positive just prior to death. However, in all but 2 of these 22 eyes, parasites were

TABLE II

Summary of Results

		Positive aspirates			Positive sections		
		At some time	At death	Lens	Anterior chamber	Posterior chamber	Vitreous
Eyes injected into anterior chamber	41						
Sacrificed 1–3 days	3	0	0	0	0	0	0
Sacrificed 5–41 days	38	27	14	22	3	6	6
Positive lenses	22	19	12	22	2	6	6
Positive aspirates, negative sections	8	8	2	0	1	0	0
Negative aspirates, negative sections	8	0	0	0	0	0	0
Total number of eyes injected into vitreous body (sacrificed 1–2 days)	5	0	0	0	0	0	2

Positive aspirates or sections means positive for *T. vaginalis*.
Negative aspirates or sections means negative for *T. vaginalis*.

not demonstrable in the anterior chamber in histologic sections, probably for technical reasons (Table II).

All of the infected lenses had lost part of their normal structure and appeared homogeneous or granular. The capsule, as a rule, was swollen and most or all of its epithelium had disappeared or was disintegrating. The capsule had ruptured at several points, often curling outward in loops.

A striking feature of the growth of *T. vaginalis* in the lens of the rabbit was the way in which the parasites ranged themselves one to 12 deep, just inside and pressing up against the capsule membrane (18 lenses) (Fig. 3). Often the parasites were the only cells in these areas. Occasionally leukocytes were widely scattered among the massed parasites. On the *outside* of the capsule, and crowded against it, leukocytes often were packed in a narrow band. At times such a packed band of leukocytes opposed an area in which the parasites were massed on the *inside* of the capsule. It was unusual to see leukocytes along the inside of the capsule even in an area free of parasites.

In 14 lenses in which *T. vaginalis* had massed against the inside of the capsule and in 5 lenses in which no such massing had occurred,

the organism had spread through the lens substance, scattered singly, in groups, or in masses (Fig. 4). In many there were inflammatory exudates in these areas. The leukocytes appeared to have invaded the substance of the lens through openings produced by breaks in the capsule. In the 22 eyes with lenses infected with *T. vaginalis,* the parasite was seen only six times in the vitreous body (Table II).

The Vitreous. Parasites were seen in sections of the vitreous in 6 eyes which had been injected into the anterior chamber. In 3 of these the lens was considerably flattened and it was difficult to determine whether the vitreous itself had been invaded or whether the organism was associated with fragments of the lens substance which had been displaced into the vitreous (Fig. 2).

Inoculation was made *directly* into the vitreous body itself in 5 eyes which were removed 1 or 2 days later. Sections disclosed a diffuse inflammatory reaction with polymorphonuclear leukocytes, mononuclear cells, and, in two instances, many parasites in the vitreous (Table II). (These findings suggest that the vitreous may be an even better site for experimental trichomoniasis than the anterior chamber. This possibility is being explored.)

Anterior Chamber Fluids

Wright's Stain of Infected Aqueous Humor Smears. Of 152 aspirations of the anterior chamber made in the 27 rabbits during the course of these experiments, 91 were positive for *T. vaginalis.* In general, when an aqueous fluid was positive for *T. vaginalis* in a wet smear, it was also positive in the stained smear. In infected aqueous fluids stained with Wright's stain the young parasites were comparatively small, clear, light-blue, oval or pear-shaped cells with large, bright red, spindle-shaped nuclei at their anterior ends, red axostyles and red flagellae (Fig. 1). They were found in small numbers in aqueous fluids infected for 2 to 6 days and were found also with older parasites in eyes infected for longer periods of time; some of these older infected eyes contained enormous numbers of parasites. The mature organisms were similar to the young parasites except that they were larger, more rounded, and had smaller nuclei. Old parasites showed disintegration of various degrees. Their axostyles and flagellae disappeared, but small, pale red, more or less spindle-shaped nuclei were still distinguishable. Some of the old parasites retained pale blue cytoplasm in which vacuoles, granular substances, and inclusions of blue and red fragments often were visible. Two nuclei could be seen in many organisms, suggesting multiplication. In a few instances some old parasites

appeared fused and six to ten nuclei characteristic of *T. vaginalis* could be seen in this group. The fluids aspirated soon after injection usually were clear and on smear only an occasional leukocyte or young parasite could be seen. As the infection progressed the fluids became thick and the smears showed leukocytes of various kinds, often degenerating; a few erythrocytes; much granular material, and sometimes fragments of lens substance.

Other Observations on Aqueous Fluids. For the 3 eyes in animals sacrificed before the fifth day, *T. vaginalis* was not found in aspirates or in sections (Table II). The parasite was demonstrated either in culture of the aqueous aspirates or in histologic sections in 30 of 38 eyes of animals sacrificed 5 to 41 days after injection. The localization of the parasite shortly after inoculation is being studied.

In 8 of the 38 eyes of animals sacrificed 5 to 41 days after injection, although the aqueous humor had been positive by aspiration at least once during infection and twice at death, no identifiable parasites were observed in sections (Table II).

In this series of 38 eyes the aspirates of the aqueous humors of 8 eyes were *always* negative for *T. vaginalis* and the eyes appeared normal grossly and histologically at sacrifice. One to five aspirations were made on each eye (Table II).

Discussion

The experiments here described suggest that the principal site of growth of *T. vaginalis* when injected into the anterior chamber is the lens. How does the lens become infected with *T. vaginalis?* There appear to be two possible explanations. The first is that the parasite may have the ability to penetrate through the uninjured capsule or through a capsule that has been injured by an inflammatory process. However, the available evidence does not support this view. As has been pointed out previously, only in a few eyes (6) have parasites been observed in sections in the posterior portion of the aqueous, and then only in small numbers. Also, one would expect to find parasites lying close to the outside of the capsule as if trying to push through it if they had the ability to penetrate the capsule. Parasites have not been seen in this position. A more plausible explanation is that the lens was inadvertently injured when the needle was inserted into the aqueous for injection or for the withdrawal of fluid. Parasites then could have invaded the lens through the points of injury. Avoiding the lens when injecting into or withdrawing fluid from the aqueous, especially if synechia was present, was not easy since the anterior

chamber of the rabbit's eye is exceedingly shallow. Furthermore, if the aqueous fluid was thick or if anterior synechia developed, it was difficult to aspirate the aqueous humor.

If *T. vaginalis* requires lens substance for growth, failure to find organisms in 8 eyes of the series could be due to the fact that the lens was not injured. At sacrifice these eyes were grossly and histologically normal.

Frequently the sections have shown no *T. vaginalis* in the anterior chamber although fluid taken just before death was positive by culture. This is not surprising since detection by smear or culture appears to be a more sensitive test than the examination of sections from which the contents of the anterior chamber had probably fallen out during processing. However, at least some of the aqueous humors which were positive for *T. vaginalis* by smear or culture, or both, may have been positive because of the presence of small fragments of infected lens substance. When the aqueous humor was thick and tenacious, the wet smear almost invariably contained lens fragments, mucoid material which might have been disintegrating lens substance, and inflammatory cells. That lens substance may be responsible for the presence of *T. vaginalis* in the aqueous seems to have been proved in one instance in which two fluids were tested from one eye. The first aspirate was clear and contained no parasites by smear or culture. The second aspirate, taken directly after the first, was cloudy and contained lens fragments, mucoid substance, and many motile *T. vaginalis*. In this one instance it appears probable that the aqueous itself did not contain *T. vaginalis* but that the second aspiration sucked up aqueous mixed with lens substance which contained *T. vaginalis*.

The attraction of the lens for the parasite, the value of the vitreous body in transmission studies, and the use of this technique for the study of other parasites and for drug evaluation remain to be studied.

Summary

Histologic observations were made on the eyes of rabbits used in the transmission of *Trichomonas vaginalis* by anterior chamber inoculation through 14 serial passages.

An affinity of *T. vaginalis* for the lens and lens substance was noted. Lenses were positive in histologic sections in 22 of 38 eyes of rabbits sacrificed 5 to 41 days after injection.

Of 152 aspirations of the anterior chamber made on 27 rabbits during the course of the experiments, 91 were positive for *T. vaginalis*.

It is probable that lens fragments were included in the material secured during some of these aspirations.

Of 5 eyes inoculated into the vitreous with *T. vaginalis*, 2 contained parasites in sections.

We are indebted to Mrs. Patricia S. Daniels for technical assistance.

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[*Illustrations follow*]

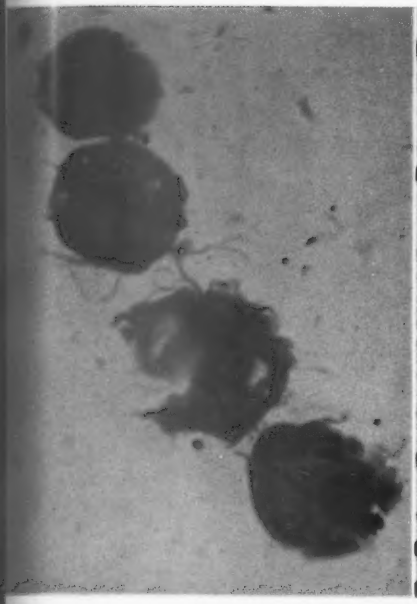
LEGENDS FOR FIGURES

FIG. 1. Rabbit died 10 days after injection into the anterior chamber of *Trichomonas vaginalis*. Aspirate of aqueous humor. Four parasites in a row show red, spindle-shaped nuclei, red axostyles, and red flagellae. Wright's stain. × 1,500.

FIG. 2. Rabbit sacrificed 2 days after injection into vitreous body. A few parasites may be seen among leukocytes in the vitreous body. Tomlinson and Grocott's stain.[15] × 490.

FIG. 3. Rabbit sacrificed 17 days after injection into the anterior chamber. A few parasites may be seen close to the inside of the capsular membrane of the lens. Wright's stain. × 950.

FIG. 4. Rabbit sacrificed 21 days after injection into anterior chamber. A few parasites are present inside the loop of lens capsule and a group of parasites may be seen farther back in the lens substance. Tomlinson and Grocott's stain.[15] × 400.



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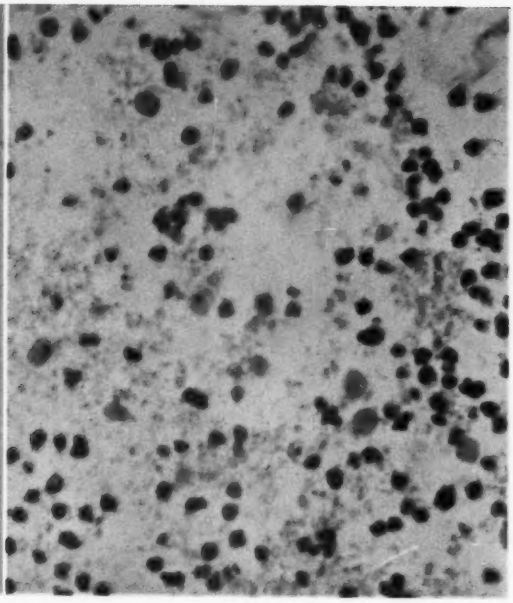

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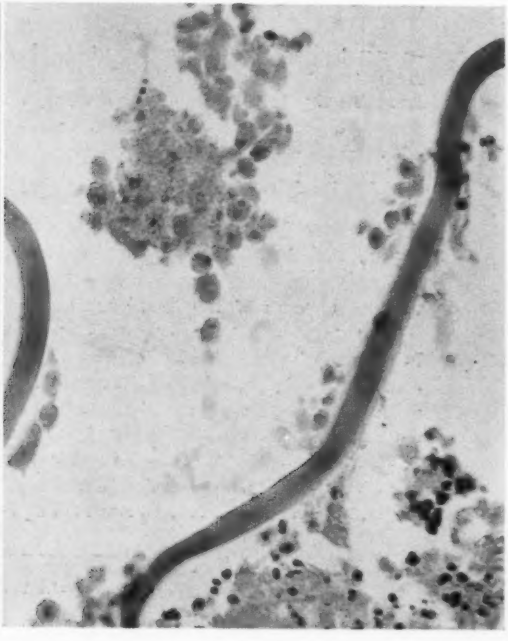

4

MORPHOTROPIC AND MORPHOSTATIC NECROBIOSIS

Investigations on Nerve Cells of the Brain *

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It is well established that anoxia† leads to rapid necrobiosis of cells and that this necrobiosis manifests itself in certain morphologic changes of the cellular structures. In nerve cells, these changes develop within a few hours after the onset of anoxia and are so characteristic that they are called ischemic nerve cell alterations. Wherever they are found, one may draw the conclusion that the respective area of the brain was already without oxygen supply before the person died.

If one considers somatic death as a permanent state of anoxia for the tissues of the body, one should expect that in every case necropsied several hours after death, all nerve cells would show more or less pronounced "ischemic" alterations. It is, however, common experience that in the majority of necropsies the nerve cells appear practically "normal," as if fixed immediately after death. In the remaining minority, they exhibit either slight deviations from the normal—the interpretation of which is often a matter of concern—or more pronounced changes, generally agreed upon to be the result of "autolysis." The above-mentioned concept of death would seem to be wrong unless it can be shown that the "autolytic" changes are identical with "ischemic" alterations of the cells and that the preservation of the cellular structure also constitutes a manifestation of necrobiosis, however different. In order to do this, it is necessary to find out which factor determines the type of cellular reaction to anoxia in a particular case. Former investigations on astrocytes[1,2] revealed that the postmortem morphologic behavior of these cells is strictly dependent upon the duration of uninterrupted hypoxia immediately preceding death. Therefore, the primary objective of the present paper is to investigate whether the same principle applies also to nerve cells.

Material and Methods

This investigation is based on animal experimentation as well as on studies of a selected group of human necropsies.

* Received for publication, December 22, 1955.

† The terms anoxia and hypoxia denote two different degrees of oxygen deficiency, hypoxia signifying insufficient supply or utilization of oxygen and anoxia, absence of oxygen supply or inability to utilize it.

Animal Experiments

The brains of 55 full-grown cats were examined. Each animal was given a single exposure to oxygen deficiency which lasted until death occurred. The duration of the exposure varied from a few seconds to about 6 hours. In 7 cats, cerebral oxygen was cut off instantaneously by decapitation. In 32 cats, oxygen deficiency was produced by lowering the barometric pressure (high altitude chamber); the remainder were exposed to carbon monoxide or nitrogen mixtures. Generally, the degree of hypoxia was rapidly increased to a critical level at which the animals were kept for varying periods. In the prolonged experiments, their clinical condition was similar to that of an agony. In some animals the intensity of the oxygen deficiency was controlled by electro-encephalogram.[1] In most animals the degree of oxygen deficiency was estimated according to Altmann and Schubothe's[3] description of the clinical manifestations of various hypoxic phases in cats. Reactions and changes in respiration were given special attention because of their significance in estimating the severity of hypoxia. After reaching a certain degree of hypoxia, the animal showed violent, uncoordinated motor reactions. It jumped up, reared, twisted, and fell down as if paretic. It became stuporous and showed panting respirations with 150 to 200 breaths per minute. As hypoxia increased, this phase of excitation was succeeded by one of motor inactivity and unconsciousness. Respiration continued to be rapid but was shallow and often interrupted by a single deep breath followed by a brief pause. From the clinical standpoint, this phase might be designated as "subcritical." When the hypoxia was kept at this level for a prolonged period or was slightly increased, the condition became "critical." It was characterized by considerable reduction of the respiratory rate (10 to 20 breaths per minute). Occasionally, a fine trembling or a twitching of the mouth or of the legs could be observed. Sometimes, this passed into a generalized epileptic attack, causing fatal respiratory paralysis. In most instances, the respiration, as in human agony, became extremely slow and shallow and eventually ceased without such an attack having taken place.

After death, the brains of a few animals were cooled to room temperature, others were kept at 32° C., and most of them at 37° C. under humid conditions. After the brains had been incubated for more than 12 hours, putrefactive bacteria interfered; therefore, some brains were placed in saline solution to which streptomycin and penicillin were added. In this way it was possible to keep portions of brain sterile in the incubator for as long as 72 hours. At certain intervals, parts of the

brain—generally one half of a hemisphere—were excised and fixed in 10 per cent neutral formalin for 1 day. The tissue blocks were washed, further hardened in 96 per cent alcohol, and finally embedded in parlodion. The nerve cells were stained with cresyl violet or thionine.

Human Cases

A total of 45 brains of adults who had not died of an internal disease were selected for this study. In 30 cases, death occurred instantaneously or within a few minutes due to suffocation, acute heart failure, hanging, severe bodily injuries, or profuse bleeding. In 4 cases, death occurred after an agony lasting for about 30 to 40 minutes, caused by bleeding or by injuries to lungs or heart. In the remaining 11 cases, agonal hypoxia lasted from 1 to 7 hours. Two of these subjects died of traumatic edema of the lungs with subsequent subacute suffocation. The others died from subacute carbon monoxide asphyxia, alcohol intoxication, or the sequelae of recent bodily injuries.

The time interval between death and fixation of the brain varied from 8 to 46 hours. The temperatures under which the bodies were kept before necropsy varied from case to case. All brains were fixed in 10 per cent formalin; the tissues from various parts of the brain were embedded in celloidin or parlodion. Cresyl violet or thionine was used for staining.

Findings in Animal Experiments

Macroscopic Observations

The brains of animals which had died within a few minutes increased in volume during their stay in saline solution, whereas the brains of animals that had been exposed subacutely to hypoxia showed neither shrinkage nor swelling. The brains of the first group became, as a rule, firm and elastic like hard rubber during the first few hours. They then continued to soften until it was difficult to cut them in the unfixed state without squashing. This softening of the tissues was very similar to the reduced consistency of an example of fresh encephalomalacia. The brains of the second group maintained their consistency and could be cut without difficulty after being in the saline solution for more than 24 hours.

Microscopic Observations

The Equivalent Picture of Nerve Cells

For determining the earliest morphologic changes of the nerve cells, it was necessary to have, for comparison, cells morphologically equivalent to their normal living condition. These "equivalent pictures"

(Nissl) were obtained by injecting formaldehyde into the arteries of a brain at the moment of decapitation. The tissue was processed in the same way as the experimental material. Figures 1 to 4 show the equivalent pictures of nerve cells of four types. The cell in Figure 1 represents the large somatochrome pyramidal cell of the third layer of the cerebral cortex. It has a centrally located light nucleus with a well marked nucleolus and scanty linin structure. The Nissl bodies are sharply outlined. Near the nucleus and at the base of the cell body they are small and somewhat irregular in shape, while in the periphery of the cell body and in the dendrites they are large, elongated, and in a somewhat parallel arrangement with ample unstained cytoplasm intervening. The medium-sized and small pyramidal cells of the cortex, as shown in Figure 2, also belong to the somatochrome type. Their nuclei are, in general, only a little smaller than those of the large cells and usually are somewhat eccentrically located. The Nissl bodies are small and granular, especially in the vicinity of the nucleus, where they are densely arranged. Toward the dendrites and, in particular, the apical dendrite, they are again elongated and separated by unstained strands of protoplasm. In nerve cells of the thalamus (Fig. 3), also representative of the somatochrome type, the rather plump Nissl bodies have an irregular shape and appear to be connected. Again, they are smaller in the vicinity of the nucleus and larger toward the periphery of the cell. Most nuclei are large and light with a well marked nucleolus. In contrast to these somatochrome types, there are nerve cells in which the nucleus is the most distinct structure, which led Nissl to designate them as karyochrome cells (Fig. 4). They are found, for instance, in the second and fourth layers of the cortex. The nuclei often show some folding of their membrane. Their linin structure is fairly distinct and the nucleolus is relatively small. The scanty protoplasm of the cell body contains small amounts of usually dust-like Nissl substance in the immediate vicinity of the nucleus or in one of the thin dendrites.

Cellular Reactions During Anoxia

Description of the microscopic changes in the experimental material will begin with those found in the brains of decapitated animals kept at 37° C. The first cellular change could be observed after about 30 minutes. The larger pyramidal cells showed a mild swelling of the cell body. The perinuclear protoplasm appeared lighter because the Nissl substance started to dissolve (tigrolysis). The more peripheral Nissl bodies were shifted toward the wall of the cell. Sometimes the

nucleus was displaced laterally. The nucleus became very pale and often showed intensified marking of its linin structure. Figures 5, 6, and 7 show this alteration in cells of the somatochrome type demonstrated in Figures 1, 2, and 3. In cells of the small karyochrome type, a similar swelling could be found; occasionally, small vacuole-like transparencies had already developed in the protoplasm (Fig. 8). This early swelling of the cells must be differentiated from the "acute swelling" (Spielmeyer) or "acute disease of nerve cells" (Nissl) seen in toxic conditions and often accompanying cloudy swelling in other organs. Besides swelling, there was occasionally a simple shrinkage of larger pyramidal cells in focal areas of the cortex, similar to that observed by Gildea and Cobb[4] in their experiments. Since Scharrer[5] was able to produce such cell shrinkage by exerting pressure on the fresh brain, it is assumed that the simple shrinkage in the present cases might have been caused by pressure on the brain at the time it was removed from the skull.

By the time the unfixed brain tissue had been in the incubator for 6 hours, considerable cellular changes had developed. They consisted of two types which, in many cells, were more or less combined. The first type was characterized by formation of "vacuoles," the content of which was not stainable. These vacuoles developed within the protoplasmic strands, enlarged, and eventually coalesced, destroying the original structure of the cell body. The nuclei usually were shrunken and triangular, with faint outlines which were often hardly discernible from the darkly staining protoplasmic remnants (Fig. 9). This type of cellular alteration was always most pronounced in the small karyochrome cells. The other type consisted of "homogenization" of the cell body in which the Nissl substance disappeared, except for occasional dust-like remnants. The cell body, usually swollen, acquired a fine granular appearance as if its protoplasm had coagulated. The nucleus either appeared unchanged, was small and hyperchromatic, or had lost its outline, leaving only an ill-defined light area containing the nucleolus and particles of the linin structure. Figure 10 shows such homogenization of a larger pyramidal cell with some vacuolization in the periphery of the cell body. It was noticed that the incidence of each type of alteration varied greatly among individual animals; in one animal, vacuolization was predominant and in another, homogenization. Perhaps the nutritive state of the animal at the moment of oxygen deprivation was significant in determining the type of alteration seen.

In tissues kept unfixed in the incubator for 12 hours, the changes

were more pronounced. The cells which had undergone a homogenizing process usually had dark, shrunken nuclei. Their protoplasm was poorly stained. There was no indication that vacuoles still would develop in such cells (Fig. 11). Those cells which suffered mainly from vacuolization during the first 6 hours of anoxia still displayed a foamy appearance, but their outlines were usually no longer recognizable (Fig. 12). Of their nuclei, only the nucleoli could be identified. In cells showing both vacuolization and homogenization, the vacuoles usually occupied the periphery of the cell bodies, and protruded toward the pericellular space (Fig. 13). Many cells showed shrinkage (Fig. 14). In contrast to the simple shrinkage caused by pressure, which has been mentioned, this process may be called ischemic shrinkage. The Nissl structure was no longer visible in these cells. Instead, the protoplasm was homogenized and often interspersed with innumerable micro-vacuoles. This shrinkage usually was accompanied by a widening of the pericellular space, obviously due to an escape of intracellular fluid through defects of the cell membrane. Alteration of this type is often described as pericellular edema. Many of the smaller, shrunken pyramidal cells exhibited corkscrew-like winding of their apical dendrites.

Beyond a period of 12 hours, the changes proceeded at a slower rate. After 48 hours, the typical picture of Spielmeyer's "ischemic" alteration of nerve cells had developed in most cells (Figs. 15 and 16). As a rule, the nuclei were shrunken, often triangular, and indistinct in outline. In some cells, however, the nucleus was either disintegrated or surprisingly well preserved. The protoplasm was homogeneous and pale, and showed signs of previous swelling or of various degrees of shrinkage. There was pyknosis or karyorrhexis of the accompanying glial nuclei. The cells which had developed extensive vacuolization were almost dissolved and only a small spot was found as the remainder of the nucleus. Those cells were better preserved in which only the peripheral protoplasm had become vacuolized (Figs. 17 and 18). In the superficial portions of the cortex which had been in direct contact with the surrounding saline solution, well marked "incrustations" of either the coarse or the finely granular type could be seen, especially in the small pyramidal cells (Figs. 19 and 20).

In portions of brains which had been in the incubator for 72 hours, the number of cell shadows was increased. Only the larger cells and the nuclei of the smaller cells could be recognized. The glial nuclei showed regressive changes everywhere.

These phenomena of structural disintegration did not proceed at the

same rate in all cells of one and the same brain. The process usually was slower in the large pyramidal cells of the cortex. Some somatochrome cells in the lower brain stem, particularly in the medulla oblongata, were remarkably resistant. Even as late as 12 hours after death, their Nissl bodies were still present. Occasionally, their nuclei were eccentrically displaced and the branching point of the axons was swollen and free of Nissl substance. Thus, the cells exhibited a picture similar to that of the initial phase of axonal reaction (Fig. 21).

In comparing the rate of cell changes in different brains, it was found to be quite the same, as long as the phase of critical hypoxia did not exceed about 10 minutes and the unfixed brains were kept at a storage temperature of 37° C. At a storage temperature of 32° C., the rate was slightly lower. It was considerably retarded if the tissues were kept at 18° C. At that temperature the first signs of homogenization and vacuolization did not become noticeable until about 12 hours after death. It was found that the type of oxygen deficiency employed did not modify the type or the mode of development of the cellular alterations.

In those animals which were exposed to a critical hypoxia for about 20 minutes before death, the cells showed also some swelling and tigrolysis but the swelling developed more gradually and tigrolysis was usually incomplete. Vacuolization, however, was rare. Cells which were fixed 3 hours after death are shown in Figure 22. The incomplete tigrolysis caused the flaky appearance of their bodies, which, in the periphery, exhibited some transparency without formation of vacuoles. The nuclei showed almost no change. After remaining unfixed in the incubator (37° C.) for 12 hours, the cellular alteration did not progress noticeably, with the exception of a few vacuoles that had formed within the protoplasmic strands between the Nissl bodies (Fig. 23).

In those cases in which oxygen deficiency had lasted longer than about 30 minutes, there was no noticeable swelling of the nerve cells. In fact, some mild shrinkage prevailed (pyknomorphia of Nissl). Usually, this condition did not change during the first 12 hours after death. Only in the smaller pyramidal cells did a certain degree of tigrolysis develop, causing a flaky appearance of the cells (Figs. 24 and 25). The nuclei often showed no pathologic reaction. Sometimes the dendrites were visible for a longer distance than normally. Cells of some types, especially those of the pallidum, showed hardly any change, whereas the nerve cells of the thalamus revealed pyknomorphia with shrunken, darkly stained nuclei.

The longer the premortal hypoxia lasted, the fewer were the changes in the nerve cells. After a premortal hypoxia of about 60 minutes, the cells fixed 30 minutes after death did not differ from their equivalent pictures shown in Figures 1 to 4. Even if kept unfixed in the incubator at 37° C. for 12 hours, they underwent no changes (Figs. 26 to 29). The presence of a few putrefactive bacteria had no influence on the cell structure (Fig. 27). In tissues kept unfixed in saline solution at 37° C. for 18 hours, the cells still showed no disintegration of their structure (Fig. 30). With increasing interval between death and fixation, however, there was a slow decrease in staining property. The general structure of the cells, however, and their nuclei remained essentially unchanged (Fig. 31). The accompanying glia showed no signs of pyknosis or karyorrhexis.

Findings in Human Brains

Macroscopic observations

In the cases of sudden death, the human brains were usually heavier than the average, weighing about 1,500 to 1,600 gm. After fixation, they were of a slightly increased elastic firmness. On cut sections, the surface of the slices was either very wet or sticky and somewhat dry. The weights of the brains of those subjects who had died after some agonal hypoxia were within the average range of about 1,350 to 1,450 gm., and their consistency after fixation was within the usual limits. On cut sections, no unusual wetness or dryness could be observed.

Microscopic observations

In the 30 human cases of sudden death, the findings were essentially the same as those observed in the rapidly killed animals. The cellular changes consisted of homogenization, vacuolization, and ischemic shrinkage. Some cases showed early, others more advanced phases of cellular alterations. The picture of homogenization was usually that represented in Figure 11. Vacuolization was most pronounced in the small nerve cells of the second and fourth cortical layers and frequently separated the nucleus from the protoplasm. The pyramidal cells of middle size also underwent vacuolization. In most instances, the histologic picture was a mixed one, showing vacuolization in one, homogenization in another, and ischemic shrinkage in a third nerve cell, side by side (Fig. 32). In the shrunken nerve cells, the apical dendrites frequently were curled in a spiral. In a few cases, changes were found which were suggestive of beginning incrustation of the pericellular structures. The glia often showed regressive changes. It

was noted that in a single case sometimes vacuolization, sometimes homogenization prevailed. As a rule, the large pyramidal cells, especially of the motor cortex, and the large motor nerve cells of the lower brain stem were better preserved than cells of other types.

In those cases in which the premortal hypoxia lasted about 30 minutes, marked shrinkage and vacuolization were absent even in the small nerve cells of the second and fourth cortical layers. The protoplasm of the cells usually exhibited a flaky appearance because of incomplete homogenization. Vacuolization was practically absent. When shrinkage or swelling were encountered, they were of a mild degree. The nuclei were often slightly smaller and darker than normal (Fig. 33).

In those cases in which death was preceded by an agonal hypoxia lasting for 1 to 7 hours, the great majority of the cells showed neither vacuolization nor homogenization, neither shrinkage nor swelling. In all somatochrome cells, the Nissl bodies were well preserved. In the smaller karyochrome cells, they had their typical dust-like structure. The nuclei usually were large and light, had a well marked nucleolus, and a delicate linin structure. In summary, the cells did not differ appreciably from those observed in cats exposed to hypoxia for more than 1 hour (Figs. 26 to 29) and from those described as equivalent pictures (Figs. 1 to 4). All cases of this group were of the same histologic appearance, although the time interval between death and fixation (35 hours in one case) and the rate of cooling of the corpse varied from case to case. The glia showed no regressive changes.

Summary of Findings

In all experimental as well as human cases in which death occurred either suddenly or within about 10 minutes after the onset of hypoxia, the nerve cells showed structural changes in the form of homogenization, vacuolization, or ischemic shrinkage. In the experiments in which the tissues were kept unfixed for various time intervals, these changes could be studied in their different phases of development. The first alteration, appearing as early as 30 minutes after death, was a swelling of the cells and beginning tigrolysis. Three to 6 hours after death, vacuolization and homogenization developed, gradually progressing to complete deterioration of the cellular structure. Forty-eight hours after death, incrustations of the pericellular structures could be observed if the unfixed tissue was kept at 37° C. At lower storage temperatures, the same cellular alterations took place at a much slower rate.

When death was preceded by critical hypoxia for about 20 to 30

minutes, only a mild swelling and incomplete homogenization took place, producing a flaky appearance of the cell body. This alteration became apparent within the first 3 hours and progressed very little during the following 9 hours. Transparencies could be seen in the protoplasm, but actual vacuolization was rare. The nuclei showed almost no changes. In human subjects who had been in severe hypoxia for about 30 minutes prior to death, the cell alterations generally were identical.

In the experimental cases in which the hypoxic phase lasted about 30 to 60 minutes, there was, from the beginning, only a mild shrinkage of the nerve cells which persisted without noticeable change during the following 12 hours.

In the experimental and human cases in which death occurred after severe hypoxia lasting for more than 60 minutes, the cells retained a structure corresponding to their equivalent picture as long as 18 hours after death, in spite of the fact that the unfixed tissues were kept at a temperature of 37° C.

Discussion

It is generally believed that in a dead body the cells are best preserved if the individual died suddenly, without having suffered from a disease, and that any development of structural changes requires the cells to have been under abnormal conditions for some time prior to death. As far as severe hypoxia is concerned, the present investigation demonstrates that just the opposite is true. The more rapidly a healthy individual dies, the more pronounced are the cellular changes which develop after death, whereas severe hypoxia lasting longer than 1 hour results in preservation of the cell structures.

How can these seemingly paradoxic phenomena be explained? Obviously, they are based on biochemical processes. If death of an individual is interpreted as a state of permanent anoxia, it should make a difference whether the cells enter this state fully supplied with the requirements for normal metabolism, as they do in sudden death, or whether their metabolism was already changed by prolonged severe hypoxia.

What occurs biochemically after rapid death? Stone *et al.*[6] found an increase in lactic acid and inorganic phosphate combined with a decrease in phosphocreatine in the brains of cats dying after breathing nitrogen for 2 to 3 minutes and showing no electro-encephalographic response for the last 20 to 30 seconds. Furthermore, it is known that, following instantaneous death, the freely fermentable sugar in the body-warm animal brain is broken down within the first 5 minutes

and a great part of the glycogen within 15 minutes. The lactic acid production increases rapidly to its maximum within 25 to 30 minutes.[7,8] About 30 minutes after death, the phosphocreatine is hydrolyzed and part of the adenosine triphosphate is broken down.[9] It is of interest that this is the same time at which the first distinct cellular alterations —a swelling of the cell body and beginning chromatolysis—can be noticed, as was observed also by Koenig and Koenig.[10] Weil[11] referred in his textbook to this "explosive change of the cellular metabolism" and concluded "that such chemical changes must result in physical changes of the structural make-up of a cell." He made this statement in discussing the course of autolytic changes but did not reach the conclusion that such changes must always occur after rapid death of a healthy individual, as proved by the present investigation.

In regard to a premortal hypoxia lasting 60 minutes or longer, no data concerning the chemistry of the tissues could be found in the literature. It is known, however, that, during a hypoxia lasting for 10 to 15 minutes, considerable lactic acid is discharged into the bloodstream and that the hypoxic brain is not able to re-utilize the lactic acid as it does under normal conditions.[12,13] From these findings it may be anticipated that, during prolonged hypoxia, other catabolic products are similarly eliminated from the tissues and, therefore, that these cells enter the anoxic state in a chemical condition different from cells after immediate death. Whatever metabolic products may remain in the tissues, they apparently have no deleterious effect on the cellular structures, at least not during the first 1 to 2 days after death.

It is of interest that mild oxygen deficiency does not bring about a discharge but only an inhibited utilization of lactic acid,[13] and that, with the restoration of normal oxygen supply, the lactic acid level promptly returns to normal and phosphocreatine is built up again.[6] This is in agreement with the observation in the present experiments that any improvement in the oxygen supply, however brief, will upset the cell-preserving effect of the preceding critical hypoxia.

The second question that arises from the present findings concerns the nature of the cellular changes occurring in the cases of sudden death. Are the post-mortem alterations of homogenization, vacuolization, shrinkage, and incrustation of pericellular structures in these cases identical with the early cell alterations in intravital lesions caused by local permanent anoxia, e.g., by an embolic occlusion of an artery? Theoretically, one should expect that the anoxia resulting from such embolic occlusion does not differ from that caused by a sudden fatal standstill of the heart in a healthy individual and that, in both in-

stances, the cells, being fully functioning when entering the anoxic condition, will gradually die. Therefore, it should be anticipated that in either case essentially the same morphologic changes take place. Indeed, in comparing the different types of early cell changes in a large area of softening (Figs. 34 and 35) with those described in the suddenly killed cats (Figs. 11 to 18) or in sudden human death (Fig. 32), one cannot find any significant difference. Even the incrustations of the pericellular structures, generally considered to be characteristic for intravital necrobiosis (Fig. 36), could be observed also as sequelae of mortal anoxia (Figs. 19 and 20). It is, therefore, more appropriate to speak of mortal necrobiosis instead of post-mortem autolysis in referring to these early changes. This does not imply that the term autolysis should be discarded. But it should be used more carefully and perhaps only for the tissue changes taking place after the phase of necrobiosis. In this way it would correspond to the term resorption used for the removal of the dead tissue by phagocytes in areas of softening. In this restricted sense, autolysis in the brain would be a rather rare occurrence in routine necropsy material fixed within several hours after death.

As to the cases in which death was preceded by prolonged critical hypoxia, it can be assumed that the cells were also still alive at the beginning of anoxia and that they, too, died gradually but without undergoing structural changes. It must, therefore, be concluded that necrobiosis need not manifest itself in cellular alterations. This leads to some interesting consequences: (1) It makes it necessary to differentiate two general types of necrobiosis, one characterized by cellular deterioration and the other, by cellular preservation. (2) Each type must be given a name reflecting its main characteristics. As has been done in regard to astrocytes,[1,2] it is proposed to call the first type *morphotropic* and the second, *morphostatic* necrobiosis. The term morphotropic necrobiosis applies not only to alterations following sudden death, but also to the milder changes in cases with premortal anoxia lasting up to about 60 minutes. (3) The finding of so-called normal cells in routine necropsy material should not be considered as "negative." The preservation of the cells indicates that they were in a certain pathologic condition prior to the individual's death and constitutes, therefore, a positive or—as paradoxic as it may sound—a pathologic phenomenon. The similarity of these "normal" cells with their "equivalent pictures," obtained by intravital fixation, is only incidental.

It is, of course, of interest to conjecture whether morphostatic

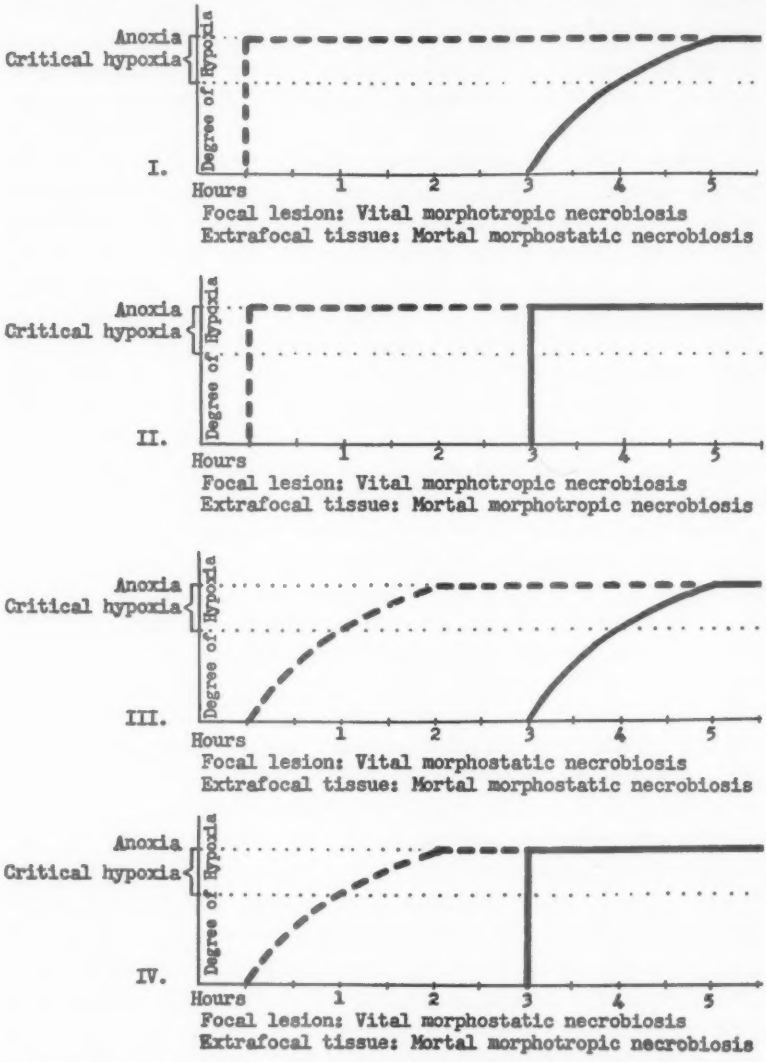
necrobiosis occurs also *intra vitam*. This cannot be proved by facts, because such a lesion would show neither gross nor microscopic "abnormalities" in its early phase, according to the present findings. It can be expected to occur occasionally in conditions with slowly progressing narrowing of the arterial lumen; e.g., in arteriosclerosis, thrombo-endarteritis obliterans, or syphilitic arteritis. Perhaps it is present in those rare cases with a history of a recent cerebrovascular accident in which the pathologist is unable to demonstrate a corresponding lesion.

A few years ago, I examined such a case in which a stroke had occurred shortly before death. At necropsy, a thrombotic occlusion of an arteriosclerotic artery was found in the area in which a lesion could be expected. Histologically, however, no definite cellular changes in the nerve cells could be seen. There was only a marked capillary stasis in the area supplied by the occluded artery. At that time, the negative finding was explained by assuming that, after the thrombosis, the focal area had received blood through arterial anastomoses inadequate for normal function of the cells but sufficient to prevent morphologic changes. But in the light of the present investigation, it can be assumed that it was possibly due to a morphostatic necrobiosis caused by a gradual development of the thrombosis.

From the experimental findings it may be concluded that such local morphostatic necrobiosis should become evident not earlier than about 48 hours after the onset of anoxia and that the first noticeable change should be a poor staining of the cell bodies with Nissl stain, while the gross shape of the cells and, to some extent, the nuclei are preserved. Such cells can sometimes be seen in a local area of the thalami in traumatic cases of 3 to 4 days' survival, in which increased supratentorial pressure had caused compression of thalamic arteries within the interpeduncular fossa without producing a softening (morphotropic necrobiosis) as it does in other cases. The final outcome of a focal morphostatic necrobiosis seems to be coagulative necrosis. In such lesions, the otherwise unstainable nerve cells become occasionally faintly visible in myelin sheath preparations and show an astoundingly good preservation of their gross structure and of their nuclei (Fig. 37). It is of interest that in these areas of necrosis no, or only abortive, phagocytosis occurs. This is, perhaps, also a sequela of the more gradual development of anoxia.

It is of interest that the similarity of the cellular alterations in morphotropic intravital and mortal necrobiosis is matched by a similarity of the gross behavior of the tissues. As could be observed, in

sudden death the entire brain first increases in volume and then very soon becomes soft just like a focal area which is suddenly deprived of oxygen. The swelling and softening were not noted in less rapid death and are therefore unlikely to occur in vital morphostatic necrobiosis.



Text-figure 1. Diagram of four quasi-standard combinations of vital and mortal, morphostatic and morphotropic necrobiosis. Broken line indicates course of intravital focal oxygen deficiency; solid line indicates course of mortal oxygen deficiency.

If it is true that not only morphotropic but also morphostatic necrobiosis occurs in intravital lesions, one must conclude that in necropsy material each of these vital necrobioses may be combined with either one of the two types of mortal necrobiosis. Therefore, four different, quasi standard combinations should be possible, as schematically illustrated in Text-figure 1.

Combination I. The focal anoxia is a sudden one, e.g., due to embolism, and death occurs after a critical hypoxia of agony of 1 hour. In a histologic slide of such a case the focal area would show deterioration of the cells whereas the cells in the vicinity of the lesions would be "normal." This combination is the most common one. It should be mentioned that the process of cellular deterioration does not stop with the occurrence of the subject's death because the local anoxia will not change whether mortal anoxia is or is not superimposed. The cellular changes in the focal area will progress postmortally as long as the temperature of the unfixed tissue is sufficiently high. This means that postponement of fixation and of cooling of the body will make it easier to identify the lesion. In determining the histologic age of a recent softening, the post-mortem progression of the cellular changes must always be taken into consideration.

Combination II. If the focal anoxia develops in the same way as in combination I but if death occurs without agony (for instance, due to sudden respiratory failure), then not only in the focal area but in the entire brain a deterioration of cells takes place. This combination is rare. Whether the focal area can be identified histologically depends on two factors: on the time interval between the onset of the focal and that of the mortal anoxia and on the time interval between death and fixation. If the focal anoxia is followed by death within 1 or 2 hours, it will be almost impossible to recognize the lesion regardless of when the brain is fixed after death. If the focal anoxia lasts for several hours, it depends on the time interval between death and fixation and on the post-mortem temperature whether the lesion can be identified.

Combination III. The focal as well as the mortal anoxia develops after a critical hypoxia lasting for 1 hour. Under these circumstances, cellular changes will not develop in either the focal or the extrafocal tissue, and it will be impossible to recognize the lesion. In this case, it does not matter when the brain is fixed after death. This combination may occur occasionally in arterial diseases leading to a slow progressive narrowing of the lumen; e.g., in arteriosclerosis, thrombo-endarteritis obliterans, or syphilitic endarteritis.

Combination IV. The focal anoxia develops slowly as in combination III but the mortal anoxia occurs suddenly. This would result in the paradoxic situation that the cells in the focal area would appear "normal" whereas the cells of the rest of the brain would undergo regressive changes. If, in such a case, the brain is fixed shortly after death, this deterioration may be too minimal to permit identification of the lesion. However, the focal area would become more evident if the fixation of the brain were postponed until the post-mortem changes become obvious. This combination can be expected occasionally in cases with supratentorial space-consuming lesions in which the circulation to certain areas of the brain was gradually reduced by compression of an artery, e.g., the posterior cerebral artery, and the patient died suddenly from respiratory failure in spite of satisfactory circulation and respiration until a few minutes before death.

Only ischemic lesions were considered in discussing these combinations. If the lesions are hemorrhagic, they can, of course, be identified at necropsy. In addition to these four standard combinations of the two extreme types of anoxia, there are further combinations possible if the intermediate types are considered.

The present investigation was limited to the nerve cells, but the principles found to be responsible for their different morphologic behavior during anoxia apply also to astrocytes and obviously to cells of other types with higher metabolism. In some of the cases investigated, the liver was examined. After sudden death, the cells showed vacuolization as described in high-altitude fatalities[14]; after more gradual death, they revealed a "normal" appearance.

Concluding, it may be emphasized that the findings of this study apply only to cases in which critical hypoxia was the sole factor that influenced the metabolism of the cells. This is relatively rare in necropsy material from hospitals. Usually, the disease of the patient has already had some effect on cellular metabolism prior to the onset of the agonal hypoxia. Furthermore, drugs stimulating circulation and respiration, when given shortly before death, may cause a temporary improvement of the hypoxic state. It is, therefore, not surprising that nerve cells with completely preserved structure as shown in the equivalent pictures are not encountered very frequently.

Furthermore, it may be pointed out that the experimental conditions in the present investigation were rather restricted, the anoxia being a permanent one and the preceding hypoxia—applied only once—being maintained without interruption. The only variable was the duration of critical hypoxia. Nevertheless, the most diverse reactions of the

nerve cells were obtained by merely varying this duration. This suggests that further variance of the experimental conditions, e.g., using different courses of hypoxia and anoxia, may answer the question why, after exposure to generalized oxygen deficiency, one case develops cortical necrosis; another, demyelination of the white matter only; a third, softening of the pallida, and a fourth, multiple hemorrhages. The type, location, and intensity of these and other sequelae in a given case may be influenced by several factors but possibly are most dependent on a specific course of the oxygen deficiency.

Summary

Starting from the concept that death of an individual constitutes a state of permanent anoxia for the cells, this study is concerned with the morphologic behavior of nerve cells during the early phase of this anoxic condition.

In animal experiments and examinations of selected human necropsy material, it was found that this behavior depends on the duration of critical hypoxia preceding the anoxia. When the critical hypoxia lasted not longer than 10 minutes, the cells underwent regressive changes such as homogenization, vacuolization, shrinkage, and incrustation of their pericellular structures. When the critical hypoxia lasted 10 to 20 minutes, vacuolization was rare, and incomplete homogenization resulted. When the critical hypoxia lasted for about 30 minutes but not longer than 60 minutes, the cells exhibited moderate shrinkage and pyknomorphia. When the critical hypoxia continued for about 60 minutes or longer, the cells showed no noticeable changes even when the unfixed tissue was kept at 37° C. for 12 to 18 hours.

The cellular alterations found in sudden death are identical with those seen in recent softenings and are therefore considered to be necrobiotic and not "autolytic" in nature.

The preservation of the cells in subacute death is also presumed to be a phenomenon of necrobiosis.

For necrobiosis characterized by cellular changes, the qualifying adjective morphotropic is proposed and for that characterized by cellular preservation, morphostatic.

This difference in the morphologic behavior of the cells is explained by a difference in the biochemical make-up of the cells while entering the anoxic state.

These findings are of obvious significance in regard to certain histopathologic problems.

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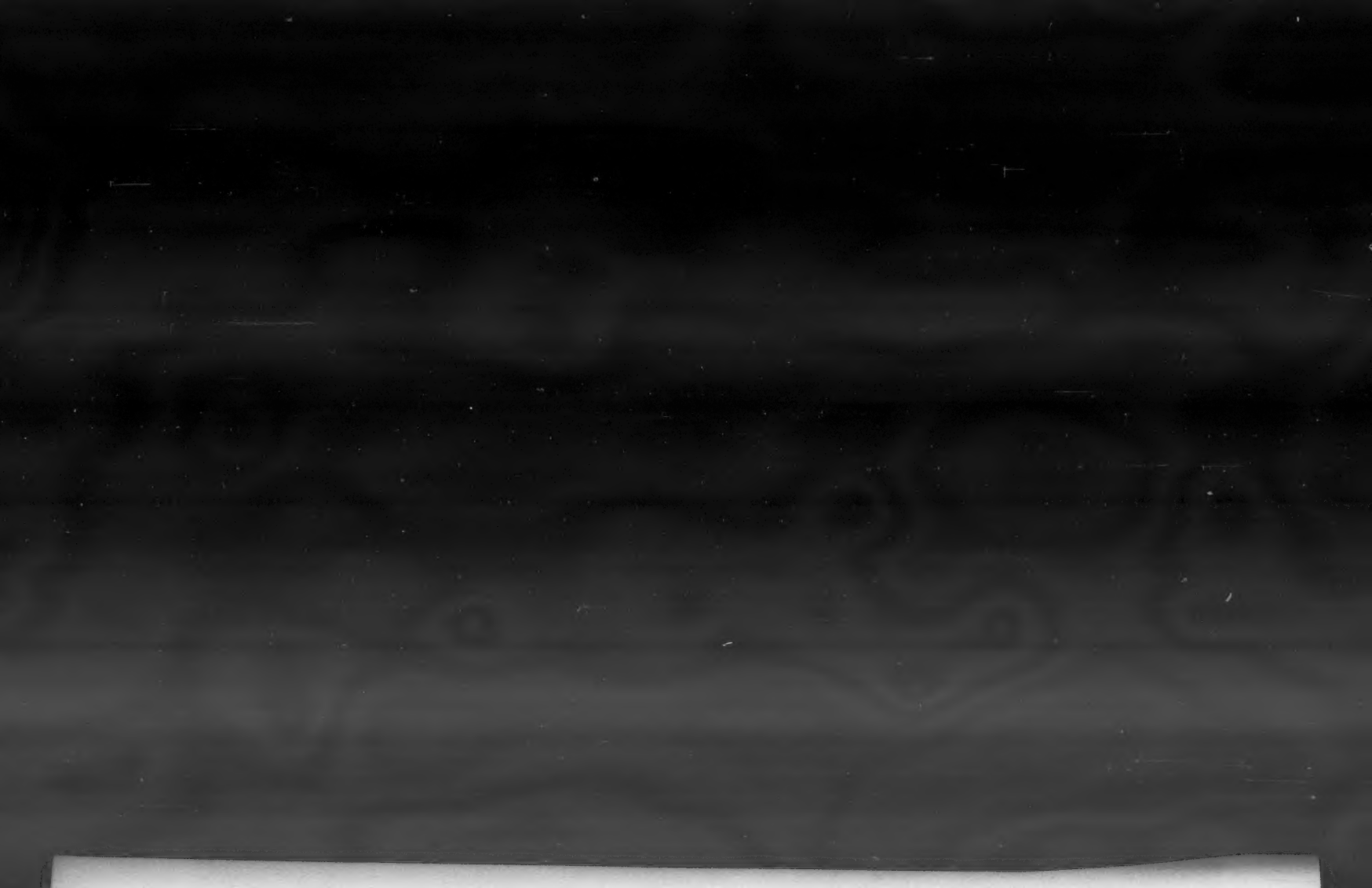
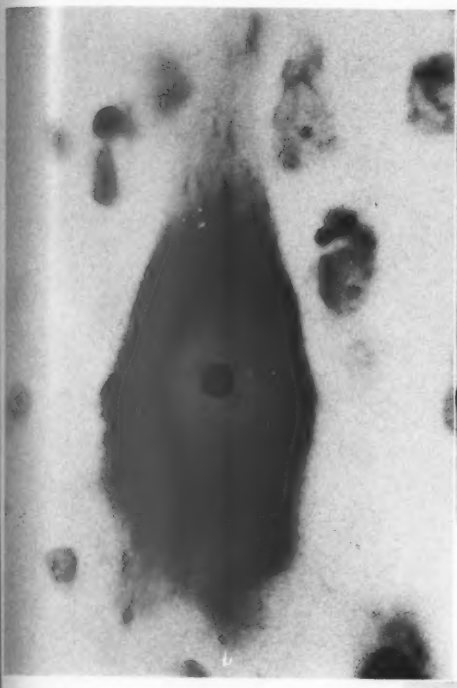
LEGENDS FOR FIGURES

FIGS. 1 to 4. "Equivalent pictures" of nerve cells of the cat obtained by injecting formalin into the arteries of the brain at the moment of decapitation. Figures 1, 2, and 3 show somatochrome cells; Figure 4, karyochrome cells. × 1,100.





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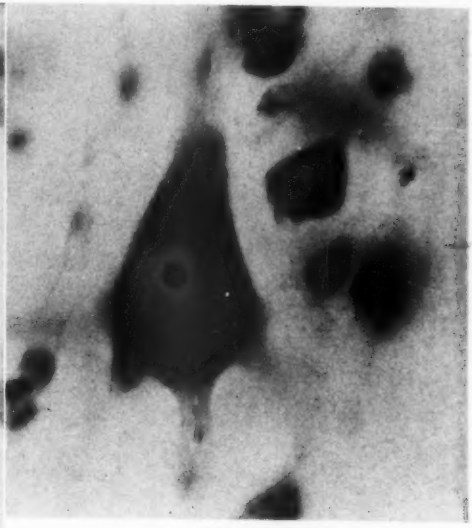

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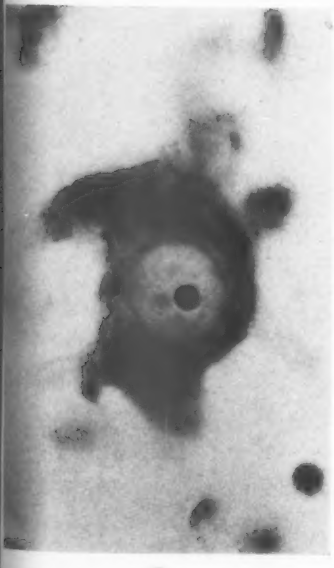

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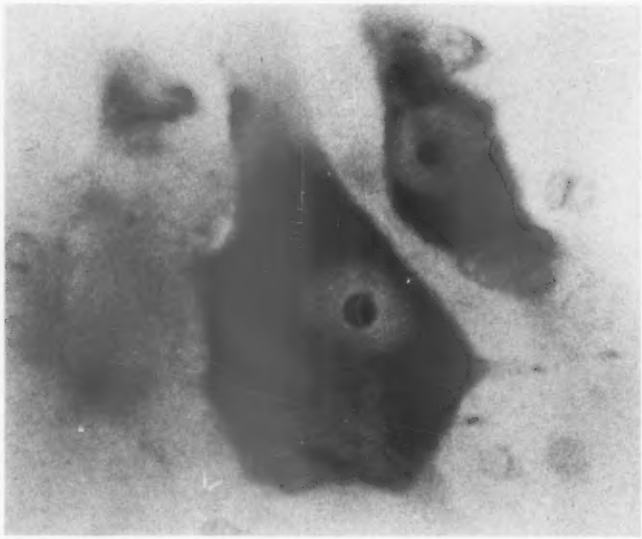
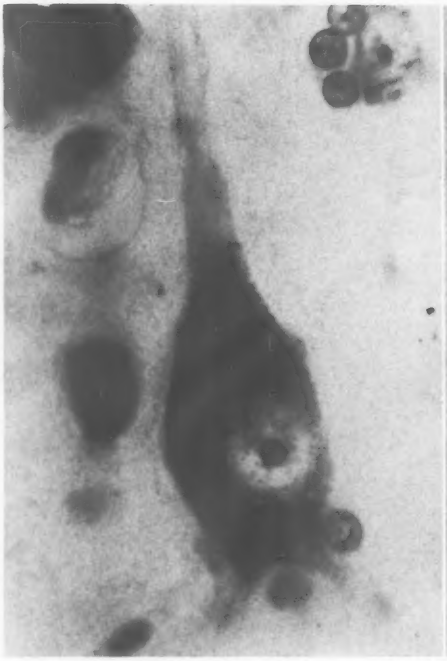
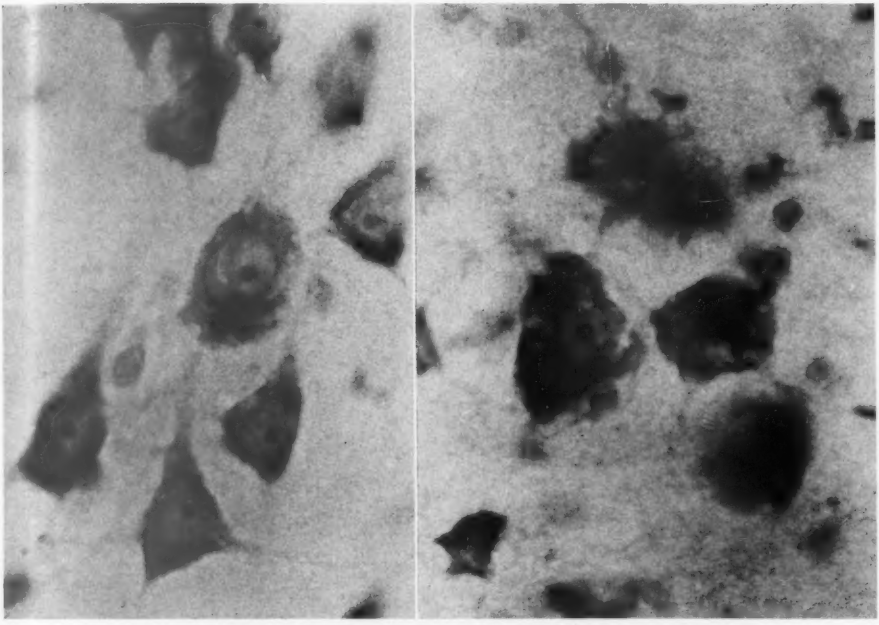
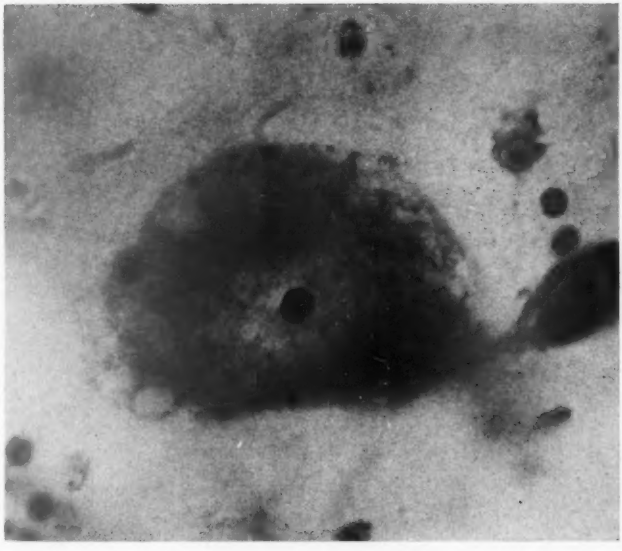




FIGS. 5 to 8. Early swelling of nerve cells of decapitated cats after the unfixed tissue was kept at 37° C. for 30 minutes. Figures 5, 6, and 7 show somatochrome cells; Figure 8, karyochrome cells. × 1,100.





FIGS. 9 and 10. Vacuolization (Fig. 9) and homogenization (Fig. 10) of nerve cells of decapitated cats after the unfixed tissue was kept at 37° C. for 6 hours. × 1,100.

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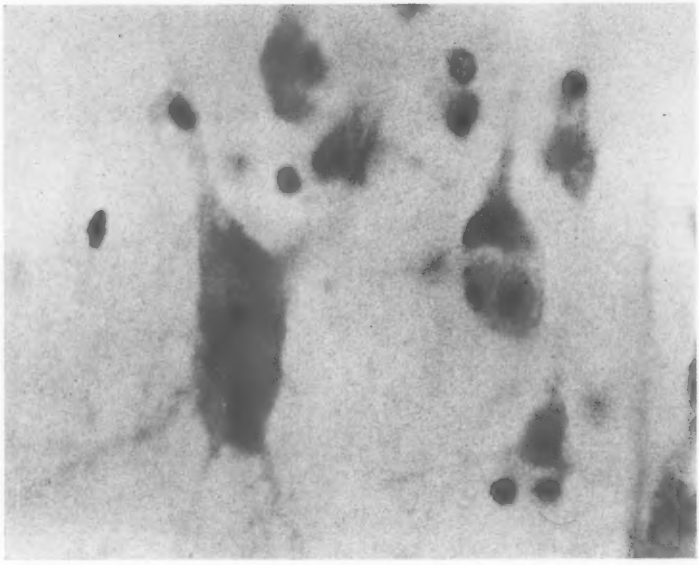
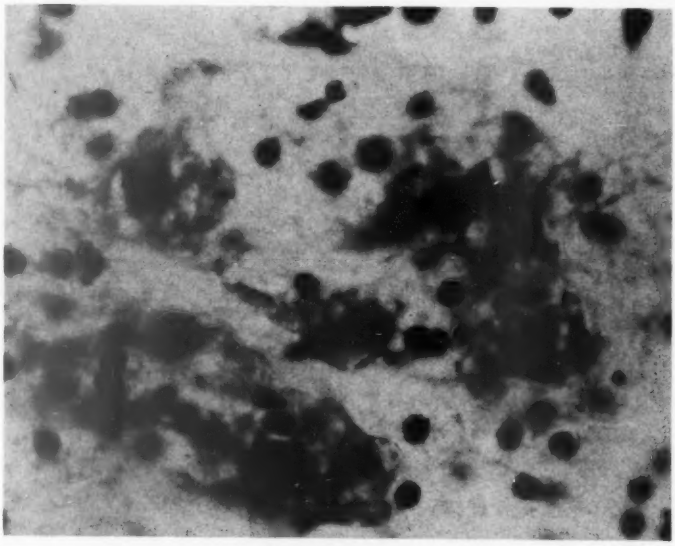


FIGS. 11 to 14. Progressive alterations of nerve cells of decapitated cats after the unfixed tissue was kept at 37° C. for 12 hours. Figures 11, 12, and 13 show vacuolization and homogenization of cells; Figure 14, shrinkage of a homogenized cell with dilation of the pericellular space at the left. Regressive changes of glial nuclei. × 1,100.



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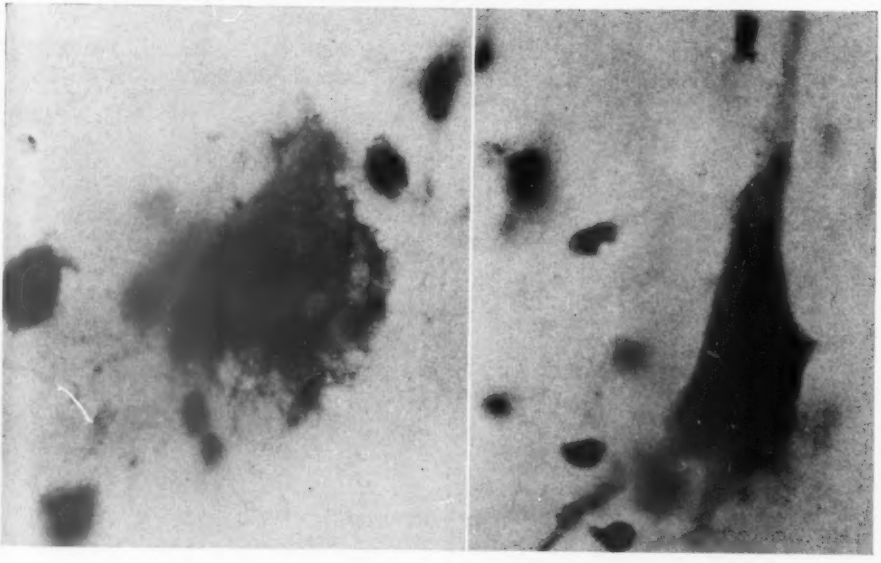

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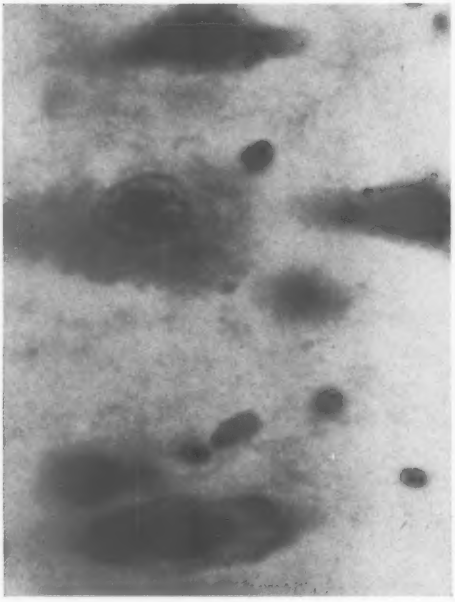

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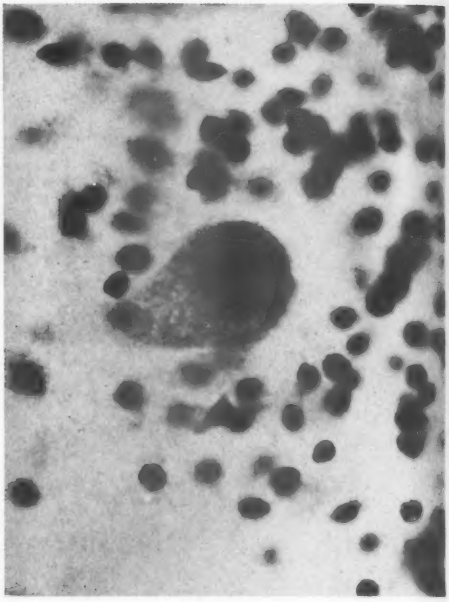

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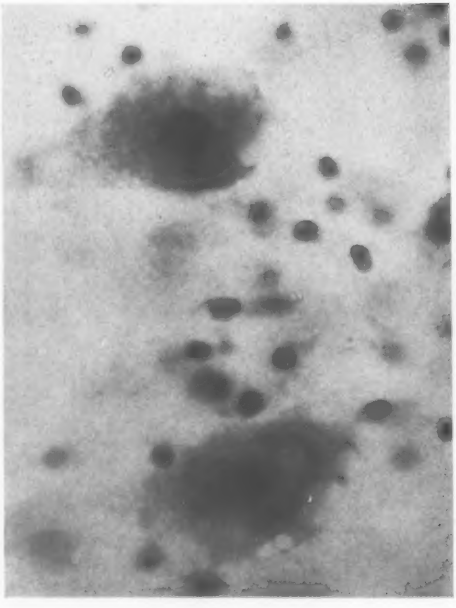

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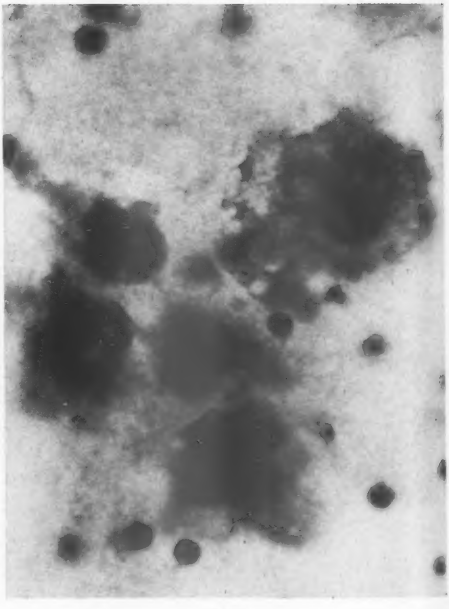

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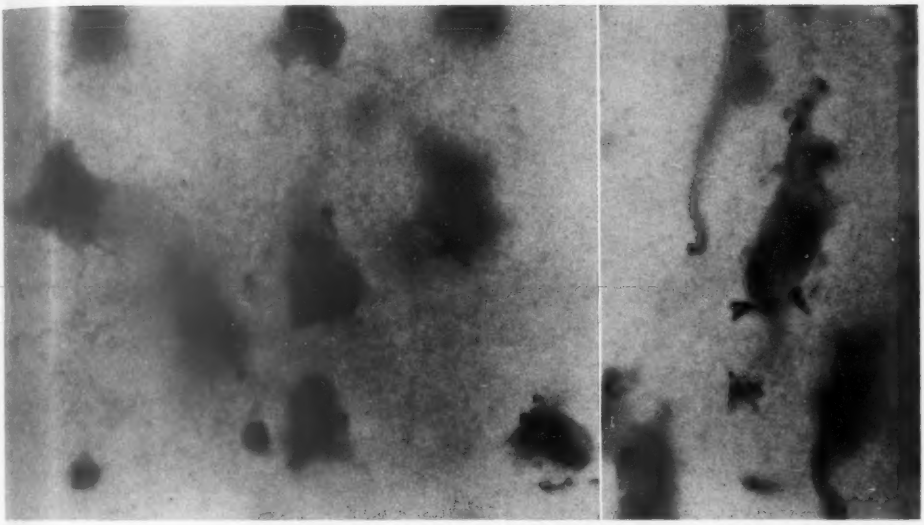

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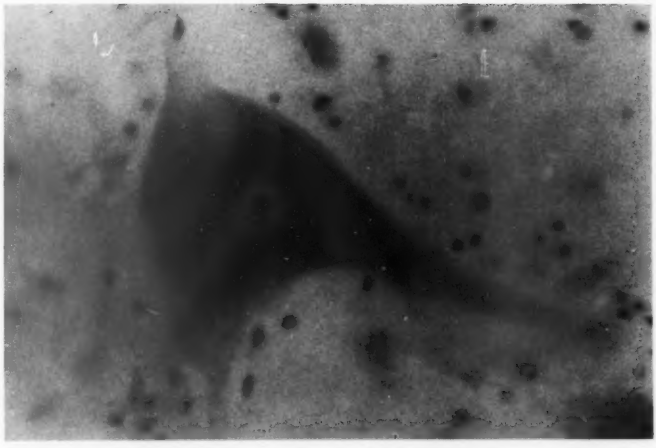

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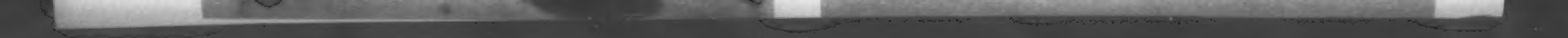
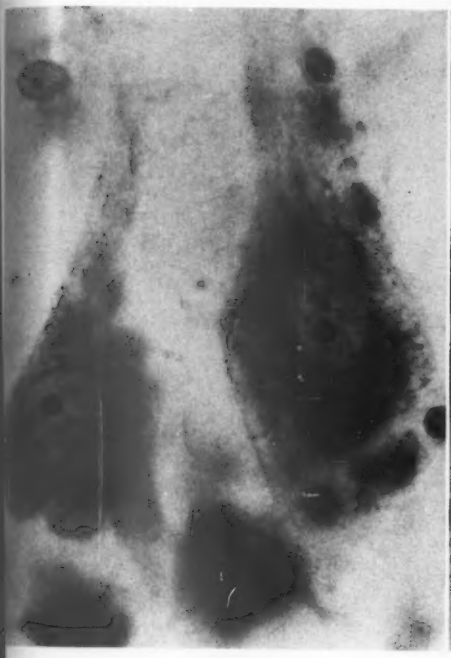
FIGS. 15 to 20. Cell changes of nerve cells of decapitated cats after the unfixed tissue was kept at 37° C. for 48 to 72 hours. Figure 15 shows progression of homogenization in pyramidal cells; Figure 16, progression of homogenization in Purkinje cell; Figure 17, vacuolization in Purkinje cells; Figure 18, vacuolization in cells of basal ganglia; Figure 19, incrustation of pericellular structures (48 hours); Figure 20, incrustation of pericellular structures (72 hours). Figures 15 to 18, × 1,000; Figures 19 and 20, × 1,100.

FIG. 21. Swelling, similar to axonal reaction, of somatochrome nerve cell of the medulla oblongata of a decapitated cat after the unfixed tissue was kept at 37° C. for 12 hours. × 1,100.

FIGS. 22 and 23. Swelling and incomplete homogenization of pyramidal cells of cats which died after exposure to critical hypoxia for 20 minutes. Figure 22 shows cells kept at 37° C. and fixed 3 hours after death; Figure 23, cells kept at 37° C. and fixed 12 hours after death. × 1,100.

FIGS. 24 and 25. Pyramidal cells of cats which died after exposure to critical hypoxia for 35 minutes. The tissue was kept at 37° C. and fixed 12 hours after death. Figure 24 shows mild shrinkage of a cell. Many Nissl bodies are still visible. Figure 25 shows mild shrinkage and mild incomplete homogenization of smaller pyramidal cells. The dendrites are traceable over a longer distance. × 1,100.





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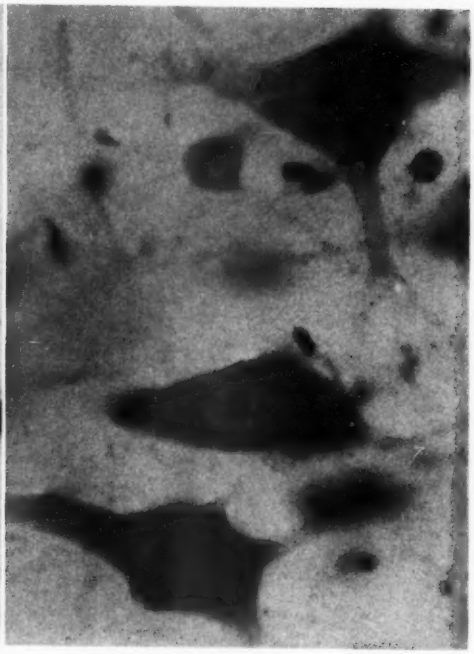

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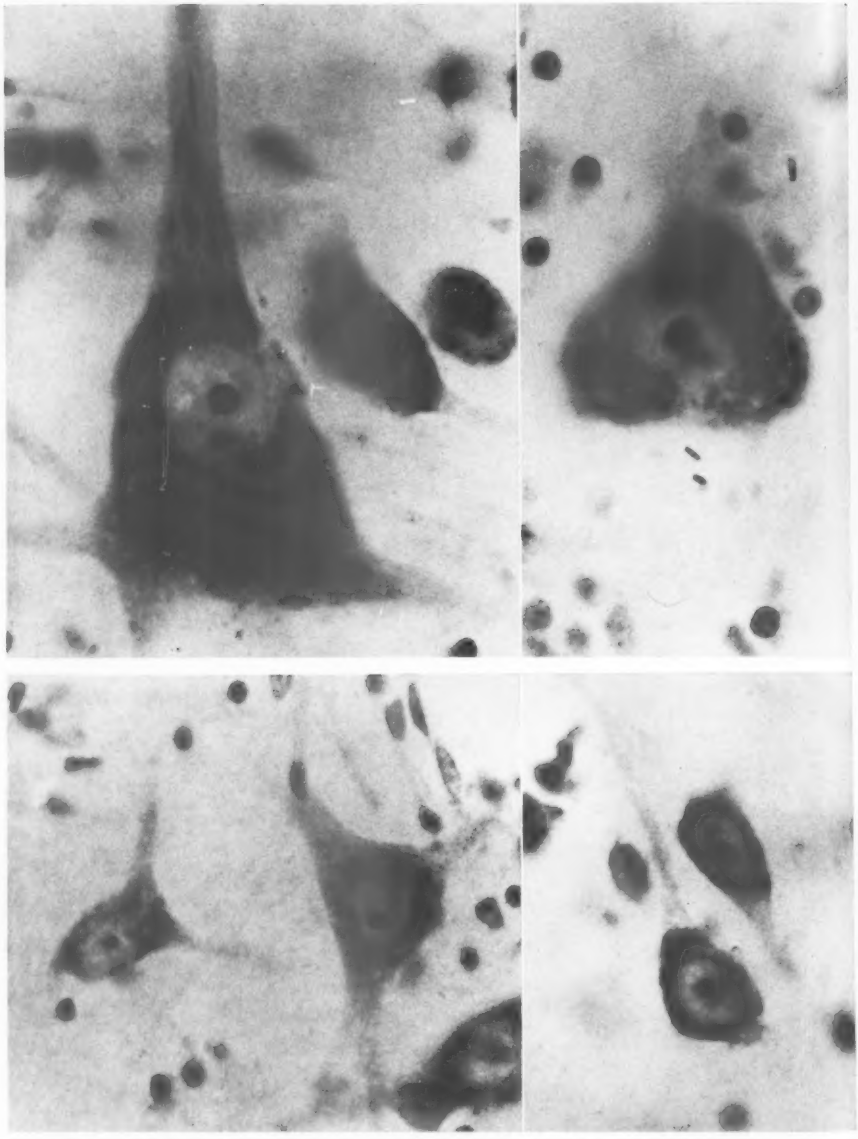
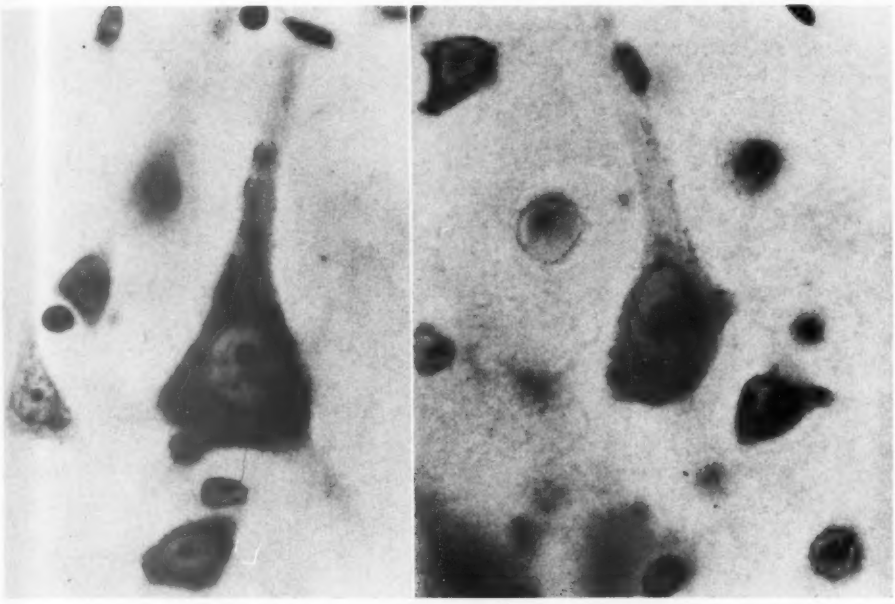
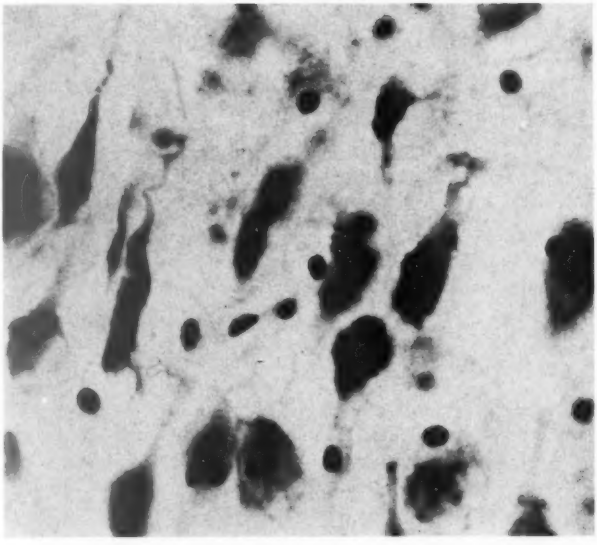


FIGS. 26 to 29. Nerve cells of four different types from cats which died after exposure to critical hypoxia for 1½ hours. The tissue was kept at 37° C. and fixed 12 hours after death. Figure 26 shows giant pyramidal cell of the motor cortex; Figure 27, thalamus cell surrounded by some putrefactive bacteria; Figure 28, pallidum cells; Figure 29, cells of the second layer of the cortex. No regressive changes of glial nuclei. × 1,100.





FIGS. 30 and 31. Smaller pyramidal cells of cats which died after exposure to critical hypoxia for 3 hours. The tissue was kept at 37° C. and fixed 18 hours (Fig. 30) and 48 hours (Fig. 31) after death. No regressive changes of glial nuclei. × 1,100.

FIG. 32. Homogenization, vacuolization, and shrinkage of human nerve cells after acute suffocation. Fixation of the brain 18 hours after death. × 1,100.

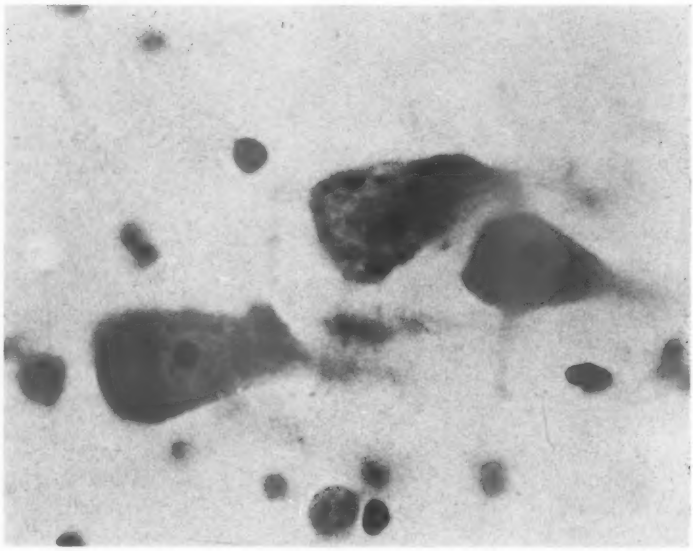
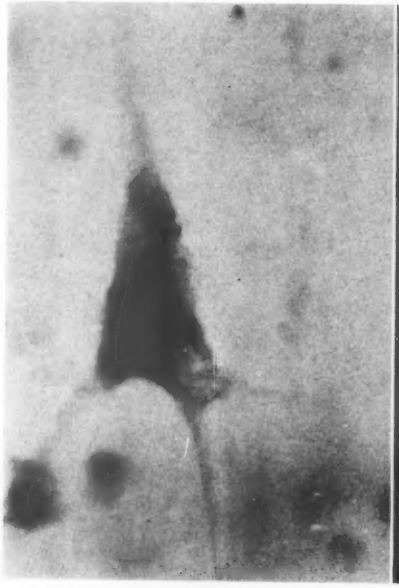


FIG. 33. Mild swelling and incomplete homogenization of smaller pyramidal cells in an individual who died after severe hypoxia lasting for about 30 minutes. Fixation of the brain 24 hours after death. × 1,100.

FIGS. 34 and 35. Homogenization (Fig. 34) and vacuolization (Fig. 35) of nerve cells from an area of embolic softening. Embolism occurred 5 hours prior to death. Fixation of the brain 20 hours after death. × 1,100.

FIG. 36. Incrustation of pericellular structures of nerve cells in an area of embolic softening. Embolism occurred about 15 hours prior to death. The brain was fixed 18 hours after death. × 1,100.

FIG. 37. Nerve cell in an area of coagulative necrosis of several years' duration, from an 80-year-old individual. The shape of the cell and the nucleus are well preserved. Protoplasm contains lipofuscin pigment. Myelin sheath stain. × 1,100.



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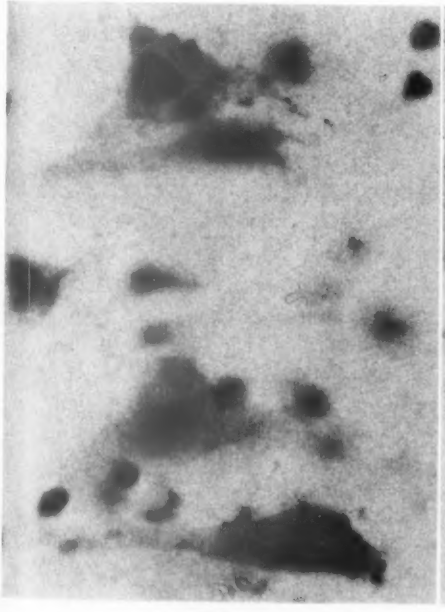

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MAST CELLS OF THE HUMAN UMBILICAL CORD *

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From the University Hospitals of Cleveland and the Institute of Pathology, Western Reserve University, Cleveland 6, Ohio

Mast cells are found in varying numbers in the connective tissue of most regions of the body, and the umbilical cord is no exception. The cells were first described in this location by Lehner,[1] in 1924, but for some unexplained reason their presence there has not been generally recognized, even in the more recent textbooks of histology.[2]

In 1950, Asboe-Hansen[3] used an alcoholic solution of toluidine blue to demonstrate granulated cells in umbilical cords from the last months of pregnancy. At that time he did not refer to these cells as mast cells, but subsequently so designated them in a review.[4] Three years later, the findings of Asboe-Hansen were confirmed by Prakken and Woerdeman,[5] who reported the presence of numerous mast cells in the umbilical cord after treatment with hyaluronidase. They used an aqueous solution of toluidine blue for staining. Asboe-Hansen[4] concluded from this work that mast cells could not be demonstrated with aqueous solutions of the dye prior to treatment of the umbilical cord with hyaluronidase. He maintained that aqueous solutions gave such an intense metachromatic background that mast cells were masked. Sundberg and co-workers,[6] in 1954, by the use of smears of Wharton's jelly and portions of cord fixed in basic lead acetate, demonstrated mast cells with toluidine blue. They pointed out that the failure to demonstrate these cells by ordinary means was probably due to the solubility of the mast cell granules in fixatives containing water.

The solubility of the granules of mast cells in fixatives containing water has long been argued. In this laboratory, neither aqueous solutions of dye nor fixatives containing water, of which basic lead acetate is one, have affected the amount of metachromatic material in mast cells. Since these cells can be demonstrated in most tissues by simple procedures, it seemed unusual that non-aqueous fixatives, basic lead acetate, non-aqueous solutions of metachromatic dye, and pretreatment with hyaluronidase were necessary. To test the requirement of these procedures and to ascertain if the mast cells of the umbilical cord differ from those in other tissues, the following procedures were followed.

* Received for publication, February 20, 1956.

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Materials and Methods

One hundred umbilical cords from full-term infants were selected for study. These had all been fixed in formalin. They were divided into five groups of twenty, covering preservation in formalin of from 1 to 5 years. A sixth group of twenty cords was fixed 24 to 48 hours before examination. Carnoy's fixative; 70, 80, 95 per cent, and absolute alcohol; and refrigeration in distilled water were used to provide umbilical cords preserved by means other than formalin for comparison.

Multiple frozen sections, which averaged 15 μ in thickness, were cut from each of these cords. They were air dried and, without further treatment, stained with an aqueous solution of toluidine blue. Throughout all procedures the concentration of the dye was 3×10^{-4} molar. The staining time was varied from 2 to 20 minutes. The sections were then mounted in water. This facilitated identification of the mast cells by preserving the deep red metachromasia of the granules.

Sections also were studied from cords fixed in formalin, dehydrated, cleared, and embedded in paraffin.

Prior to staining, some of the mounted frozen sections were treated with absolute alcohol, benzene, or xylene for 24 hours.

Results

Mast cells were identified in all preparations. The ease with which they were recognized was related directly to the length of staining time, periods of 2 to 10 minutes giving the best results (Fig. 1). When the time was increased, it became more difficult to identify the cells because the staining of the background tissue increased and the contrast between mast cells and the surrounding tissue diminished.

The cells were usually round or oval and well defined, with a central or slightly eccentric nucleus. The cytoplasm was completely filled with a metachromatic, coarsely granular mass when mounted in water (Fig. 2). In such preparations most of the granules were not discrete, and they were never identified outside the cell borders.

The amount of granular material and the degree of metachromasia of the mast cells preserved in formalin for 5 years varied little from those of cells fixed for 24 hours. Umbilical cords placed in Carnoy's fixative; 70, 80, 95 per cent, and absolute alcohol; or refrigerated in distilled water gave results similar to those obtained after formalin fixation. In the sections which had been pretreated in absolute alcohol, benzene, or xylene and in those from paraffin blocks the amount of metachromatic material present within the mast cells was decreased. No effect was apparent when frozen sections were passed through

descending concentrations of alcohol at the usual intervals used for hydration.

When stained preparations were dehydrated in alcohol or air dried, the metachromatic material was clumped around the nucleus as a purplish red, non-granular mass. When these sections were then mounted in a medium such as permount,* technicon,* or stacol,* the granules were apparent and most of them well separated (Fig. 3). Only when sections had been mounted in this manner were granules found in an extracellular position. The cytoplasm was again filled with a red mass in which the granules were not well defined, when the sections were rehydrated to water. The granules previously seen outside the cells remained extracellular. Lack of definition of the granules in aqueous mounts was due in part to faulty optics. If the coverglass of watery mounts was covered with immersion oil or mounting medium, the granules within the cells were much better defined.

Mast cells were usually in greater numbers around the vessels and at the periphery of the cord, but they were present also in the fibrous stroma and within areas of Wharton's jelly. There was considerable variation in the number of mast cells in specimens from different cords and even from the same cord. This might range from 6 to as many as 100 cells in adjacent sections.

Discussion

The findings are in accord with those of others who have described mast cells in the umbilical cord. However, the specific conditions which have been considered essential for the demonstration of mast cells (non-aqueous fixative, basic lead acetate, non-aqueous solutions of metachromatic dye, use of hyaluronidase) were found to be unnecessary. Instead, the critical factors were found to be a dilute solution of the dye and a relatively short staining period.

There was a decrease in the amount of metachromatic material in the mast cells after prolonged stay in alcohol, benzene, or xylene, and following the dehydration and clearing procedures prior to embedding. This suggests that a portion of this substance is removed by these solvents. In contrast to this, there was no diminution in the amount of this material when water or water-containing fixatives were used.

The cytologic characteristics of the cells also could be varied by technical procedures. When well hydrated, they contained a coarsely

* Permount, Fisher Scientific Co., Pittsburgh, Pa.; technicon, Technicon Co., Chauncey, N.Y.; stacol, no source currently known to the author.

granular red mass, which completely filled the cytoplasm. Dehydration produced a condensed, purplish red mass which occupied only a part of the cytoplasm. This change to a more condensed mass from hydration to dehydration would fit well with the hydrophilic nature of the polysaccharides. When the cells were viewed through media of increased density, the granules were more distinct from one another in both hydrated and dehydrated specimens.

Individual granules were identified outside of the cells only when mounting media, such as permount, technicon, or stacol, were used.

Summary

Mast cells are numerous in the umbilical cord. The critical factors for the demonstration of these cells in the umbilical cord are the concentration of dye and the length of time of staining. Their metachromatic granules are not appreciably affected by either prolonged stay in fixatives containing water or staining in aqueous solutions. The cytologic characteristics of these cells can be altered by hydration, dehydration, and changing the media through which they are viewed.

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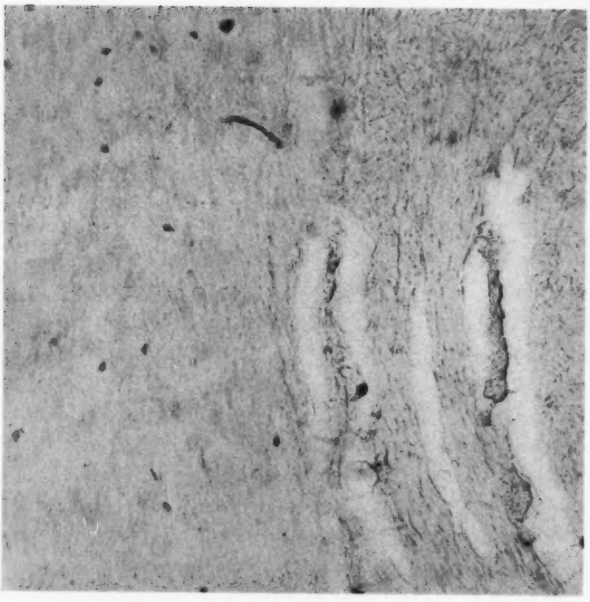
LEGENDS FOR FIGURES

FIG. 1. Section of umbilical cord fixed in formalin and stained for 2 minutes in aqueous toluidine blue and mounted in permount. This illustrates the intense metachromasia of the mast cells and their distribution adjacent to the umbilical vein, the lumen of which is at the right. × 90.

FIG. 2. Mast cell mounted in water. × 2,400.

FIG. 3. Mast cell mounted in permount. × 2,400.



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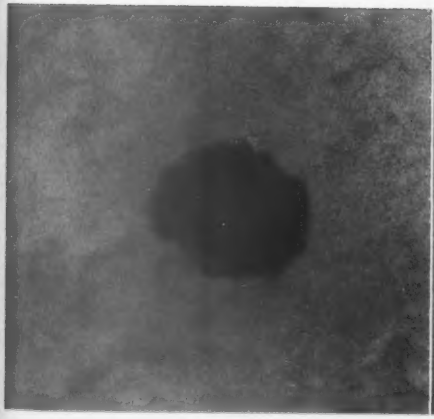

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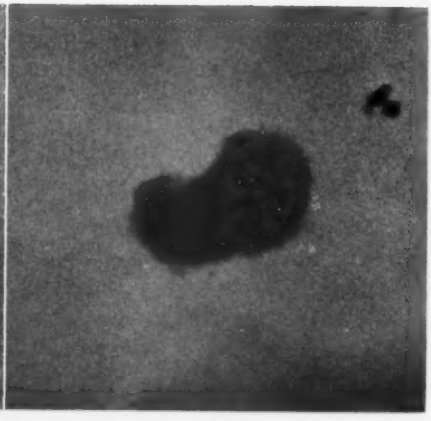

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THE NATURE OF ARTERIOLAR AND CAPILLARY OCCLUSION IN PATIENTS WITH CARCINOMA *

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Vascular and cardiac lesions, such as migratory phlebitis, arteriolar and capillary thrombosis, and degenerative verrucal endocardiosis (indeterminate or thrombotic non-bacterial endocarditis) are observed occasionally in patients with carcinoma. However, their nature, pathogenesis, and exact relationship to the neoplasms accompanying them have not been resolved. Many examples of migratory phlebitis in patients with carcinoma, particularly of the pancreas, stomach, lung, and gallbladder, have been recorded since Trousseau's observations in 1860[1] concerning this association.

It has been postulated that thromboplastin, mucins, or proteolytic enzymes elaborated by these neoplasms might be responsible for venous thrombosis, since, experimentally, they are capable of inducing coagulation. However, Durham,[2] in his recent review of this subject, has failed consistently to note any alterations in the usually measured factors of the coagulation mechanism in such patients. On the other hand, Korst and Kratochvil[3] have demonstrated a cryoprotein possessing many of the physical properties of fibrinogen in the blood of a patient with migratory phlebitis and bronchogenic carcinoma. This observation indicates the need for further exploration of the coagulation mechanism or of the presence of unusual thrombogenic substances within the blood of patients with this syndrome, before its rôle in the pathogenesis of this lesion can be dismissed. Histologic studies, although few, have failed to reveal unusual alterations within affected vessels which might offer some insight into underlying pathogenic mechanisms.[4,5]

The arteriolar and capillary lesions accompanying carcinoma have received less attention than those of large veins, apparently because of their less frequent occurrence. Some investigators[6] have considered the possibility that the intra-arteriolar lesions might represent emboli from the verrucal endocardiosis also noted in patients with carcinoma. More recently, disseminated intra-arteriolar and capillary occlusion in patients with carcinoma has been considered an example of the so-

* Received for publication, March 31, 1956.
Presented at the Fifty-third Annual Meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, April 28, 1956.

called generalized intravascular coagulation syndrome.[7-9] Besides occurring in patients with carcinoma, it has been described in infants with enteritis due to the O 111 B_4 strain of *Escherichia coli*,[10] with incompatible blood transfusions,[11] and in association with such obstetric problems as bilateral renal cortical necrosis, hepatic and pituitary necrosis, and abruptio placentae.[12] In addition, it has been likened to the generalized Shwartzman reaction [10,12] observed in rabbits following preparatory doses of various bacterial endotoxins and the provocative administration of endotoxin or such non-bacterial substances as kaolin, starch, glycogen, and acidic polymers of high molecular weight. The ability to induce the generalized reaction in pregnant rabbits with only a single dose of endotoxin[13] tends to indicate further the similarity between this phenomenon and the so-called generalized intravascular coagulation syndrome observed in pregnancy. The clinical manifestations of this syndrome have included shock, a hemorrhagic diathesis, pseudomembranous enteritis, acute renal failure, and verrucal endocarditis. The common denominator of the foregoing has been described as thrombocytopenia, prolongation of prothrombin time, fibrinolysin activation, and fibrinogenopenia due to the presence of disseminated intravascular fibrin thrombi.[9,10] In patients with carcinoma this latter development has been interpreted as resulting from the elaboration of thrombogenic substances by the neoplasm.[9] It is pertinent to note that verification of the exact nature of these intra-arteriolar lesions has not been presented.

Recently we have had the opportunity to study the tissues from two patients with carcinoma—one bronchogenic and the other arising in the stomach. In the former, all of the cardiac and vascular lesions known to accompany carcinoma, as have been enumerated, were present. Only arteriolar lesions were observed in the patient with gastric carcinoma. It was considered advantageous to investigate the nature of these cardiac and intra-arteriolar lesions (the so-called generalized intravascular coagulation syndrome) by various histochemical and histologic techniques. The results have confirmed our original impression, gained from examination of sections prepared with hematoxylin and eosin, that the lesions were not fundamentally fibrin thrombi, but represented a primary alteration of the walls of arterioles and, in some instances, of small arteries. Further significance of these findings became apparent when it was learned that many of the clinical and hematologic features of the so-called generalized intravascular coagulation syndrome were present in the patient with bronchogenic carcinoma, whereas, in the patient with gastric carcinoma, in whom

the lesions were less severe or less completely developed, these features were not present. A different concept of the pathogenesis of the so-called generalized intravascular coagulation syndrome in these patients with carcinoma evolved from these findings.

Report of Cases

Case 1

A white man, 34 years old, was admitted to the hospital complaining of cough, weakness, and the loss of 40 lbs. in weight. Examination revealed both saphenous veins from the ankle to the knee to be firm, cord-like, and tender. A grade 1 systolic murmur was heard over the apex of the heart. Red blood cell counts varied from 2.9 to 3.4 millions per cmm. and hemoglobin from 9 to 10 gm. per 100 ml. There were 0.8 per cent reticulocytes. The leukocyte count varied from 10,000 to 12,000 per cmm., with a normal differential. Platelets dropped from 206,000 per cmm. on admission to 70,000 1 week before death. Prothrombin times and prothrombin consumption tests were normal except during anticoagulant therapy. Coagulation and bleeding times also were within normal limits. Cold agglutinins were not demonstrable within the serum and an "L.E." preparation was negative. Total serum proteins were 6.5 gm. per 100 ml. of blood with 2.7 gm. of albumen and 3.8 gm. of globulin. X-ray examination of the chest revealed a soft infiltration of the right apex and a denser shadow in the left hilum. An electrocardiogram revealed non-specific "T" wave changes. The patient's course was characterized by the development of generalized petechiae, migratory phlebitis, and a systolic murmur of moderate intensity over the aortic region. The liver became palpably enlarged and a needle biopsy revealed congestion. Immediately following this procedure the patient developed a shock-like state. Although two transfusions of whole blood restored his blood pressure, his general condition deteriorated and he succumbed 2 weeks following liver biopsy and 2 months after admission to the hospital.

Necropsy Findings

Gross Examination. A mucinous tumor, measuring 6 cm. in diameter, was found arising from the anterior branch of the right upper lobe bronchus, with infiltration of the middle and upper lobes of the right lung. A similar tumor nodule measuring 1.5 cm. in diameter was noted beneath the pleural surface of the apex of the left lung. The heart weighed 325 gm. Its pericardial surface was covered by fibrinous exudate and there were several discrete tumor nodules measuring up to 7.5 mm. The myocardium of the anterior portion of the interventricular septum and apex was soft and yellow-tan. Small areas of fibrosis also were noted within the myocardium. Mural thrombi were found adherent to the endocardium at the site of myocardial discoloration. Aortic and mitral valves bore confluent, pink, variegated vegetations (Fig. 1) on their ventricular and auricular surfaces, respectively. The coronary arteries were without alteration, except that the left was completely occluded at 3 cm. from its origin by material appearing similar to that comprising the aortic and mitral vegetations (Fig.

2). Old and recent infarcts were apparent in the spleen and kidneys, and the liver was markedly congested. There were superficial ulceration and hyperemia of the mucosa of the jejunum. The superior mesenteric and splenic arteries were occluded by material similar to that noted within the left coronary artery and comprising the vegetations of the aortic and mitral valves. The left iliac vein contained a large antemortem clot.

Microscopic Examination. Neoplasm. Sections of tumor fixed in Zenker's acetic fluid and stained with hematoxylin and eosin revealed a malignant epithelial neoplasm comprised of columnar and polygonal cells of large size, occasionally forming atypical lumina. Reduplication of the latter was not uncommon. In some areas tumor cells were arranged in sheets and masses. Their nuclei were large and for the most part hyperchromatic with prominent nucleoli. Mitotic figures were frequent. The cytoplasm of the cells was eosinophilic and homogeneous, although signet-ring forms and intracellular vacuoles were apparent, particularly in those areas in which an alveolar arrangement was not conspicuous. Sections stained by the periodic acid-Schiff method revealed the vacuoles as well as the lumina to contain markedly reactive material, apparently mucin. Similar neoplastic tissue was observed in sections of the left lung, adrenal glands, mediastinal and peribronchial lymph nodes, thymic remnant, pericardium, and peri-esophageal tissues.

Vascular Lesions. Sections of the lung, myocardium, spleen, testis, kidney, and skin, stained with hematoxylin and eosin after fixation in 10 per cent formalin as well as in Zenker's acetic fluid, revealed many of the arterioles and, on occasion, the capillaries and small arteries to be occluded by homogeneous and granular acidophilic "thrombi" (Fig. 3). The walls of affected vessels in some instances also contained a similar material. This intramural alteration also was noted in vessels which did not reveal luminal occlusion, and various stages of apparent herniation of this material into vascular lumina could be discerned (Fig. 4). An intact endothelial surface was observed covering many of the "thrombi" formed in this manner. Inflammatory cells were not present. Sections of the kidneys revealed occlusion of the capillary loops by this homogeneous, eosinophilic substance, in addition to the arteriolar lesions noted. Sections of skin from purpuric areas revealed escape of red blood cells into the surrounding corium in areas of vascular damage. The occlusive material within the coronary, splenic, and mesenteric arteries, and the vegetations on the mitral and aortic valves were, for the most part, comprised of homogeneous eosinophilic and

finely granular material similar to that noted within the walls and lumina of the arterioles and small arteries. Their outer surfaces, however, were laminated and fibrillar. The mitral and aortic valves were slightly edematous and contained foci of eosinophilic homogeneous material as has been noted. On the other hand, the thrombi observed grossly in the ramifications of the pulmonary artery and iliac vein were comprised of laminated and fibrillary masses of eosinophilic material containing red blood cells and leukocytes. Alterations of the vascular walls in these instances were not noted, although beginning organization at sites of attachment was evident.

Case 2

A white man, 66 years old, was admitted to the hospital complaining of nausea, vomiting, hematemesis, dysphagia, and a 24 lb. weight loss during the preceding 4 months. Examination revealed a non-tender mass in the epigastrium. He had mild microcytic hypochromic anemia. Bleeding and coagulation times, as well as platelet counts, were within normal limits. Roentgenograms of the stomach revealed a large filling defect in the gastric wall and lower portion of the esophagus. His condition progressively deteriorated and he died 3 weeks after admission to the hospital.

Necropsy Findings

Gross Examination. The heart weighed 200 gm. The myocardium was brown and contained small foci of fibrosis in the interventricular septum. The valves were free of vegetations. Examination of the stomach revealed a fungating, ulcerated tumor replacing the entire mucosal surface of the body and cardia with extension into the lower esophagus. Metastatic tumor replaced approximately 50 per cent of the hepatic parenchyma.

Microscopic Examination. Neoplasm. Sections of stomach stained with hematoxylin and eosin after fixation in Zenker's acetic fluid revealed a malignant epithelial neoplasm formed by large columnar and polygonal cells having an alveolated structure, as well as being arranged in sheets and masses. Nuclei were, for the most part, large, vesicular, and hyperchromatic. Frequent mitotic figures were encountered. The cytoplasm was usually homogeneous and eosinophilic, although signet-ring forms and intracellular vacuoles were not uncommon. Sections stained by the periodic acid-Schiff method revealed intracellular mucin as well as pools of mucin within atypical lumina. Similar tumor was observed within the lymphatics and small vessels of the lungs, and in the liver, spleen, pancreas, bone marrow, adrenal glands, and perigastric, periaortic, and supraclavicular lymph nodes.

Vascular Lesions. Sections of heart, small intestine, and spleen prepared from tissue fixed in 10 per cent formalin and Zenker's acetic

fluid revealed focal accumulations of homogeneous, eosinophilic material unaccompanied by inflammatory infiltration in the walls of arterioles and small arteries similar to that observed in case 1. On rare occasion protrusion of the material into vascular lumina was observed, although complete occlusion was not seen. An intact endothelial surface was present covering the protruding material in most instances. In the myocardium these vascular lesions occasionally were noted adjacent to areas of myocardial fibrosis.

Special Studies

In an effort to determine the nature of the valvular and vascular lesions encountered in case 1 and the arteriolar lesions of case 2, sections prepared from tissue fixed in 10 per cent neutral formalin and infiltrated with paraffin in the usual manner were prepared and stained by the following procedures. In addition, control sections from a rheumatoid nodule and examples of renal and splenic amyloidosis were similarly prepared and stained simultaneously. The methods employed were those described by Lillie[14] unless otherwise indicated.

1. Periodic acid-Schiff's procedure (PAS).
2. Phosphotungstic acid hematoxylin (PTAH).
3. Masson's trichrome method.
4. Thionine, pH 4, 0.05% for ½ hour. All sections examined after alcohol differentiation.
5. Wilder's reticulum method.
6. Crystal violet.
7. Digestion with trypsin (Difco Lot no. 428446) in phosphate buffer, pH 7.6 for 2, 4, and 6 hours at 37° C. followed by methods 1 and 2. Sections were collodionized following treatment with trypsin prior to staining.
8. Orcein elastic tissue method.
9. Verhoeff-van Gieson's elastic tissue method.
10. Bennhold's Congo red method for amyloid. Sections were differentiated in alcohol until connective tissue and background were colorless.
11. Dunn-Thompson technique for hemoglobin.

The results are listed in Table I.

Comment

The results of the tinctorial studies listed in Table I indicate the similarity to fibrinoid of the material comprising the arterial, arteriolar, and endocardial lesions in these patients with carcinoma. Unlike fibrin, it is argyrophilic and markedly resistant to tryptic digestion (Figs. 5, 6, and 7). In these respects it is identical to fibrinoid as characterized by Glynn and Loewi[15] and also noted by one of us.[16] The nature of this material is in accord with the morphologic observations concerning the initial site of the lesions and the subsequent de-

TABLE I

Comparison of Staining Reactions of the Vascular and Valvular Lesions in Cases of Carcinoma with Fibrin, Fibrinoid, and Amyloid*

Procedure	Arteriolar and capillary lesions	Fibrinoid	Fibrin	Amyloid	Mitral and aortic vegetations	Coronary, splenic, and superior mesenteric	Pulmonary artery and iliac vein
Tryptic digestion	Unaffected†	Unaffected	Digested	Unaffected*	Unaffected†	Unaffected†	Digested
Silver	Argyrophilic	Argyrophilic	Uncolored	Faintly argyrophilic	Argyrophilic	Argyrophilic	Uncolored
PTAH	Orange and blue*	Orange	Blue	Orange–red	Orange–blue*	Orange–blue*	Blue
PAS	Red–purple	Red–purple	Red–purple	Red–purple	Red–purple	Red–purple	Red–purple
H and E	Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic
Thionine	Orthochromatic	Orthochromatic	Orthochromatic	Orthochromatic	Orthochromatic	Orthochromatic	Orthochromatic
Crystal violet	Metachromatic?	Metachromatic?	Metachromatic?	Metachromatic	Metachromatic?	Metachromatic?	Metachromatic?
Trichrome	Purple	Purple	Red–purple	Red–purple	Purple	Purple	Purple
Congo red	Uncolored	Uncolored	Uncolored	Red	Uncolored	Uncolored	Uncolored
Orcein‡	Tan	Tan	Tan	Tan	Tan	Tan	Tan
Verhoeff–van Gieson‡	Gray–brown	Gray–brown	Gray–brown	Gray–brown	Gray–brown	Gray–brown	Gray–brown
Dunn–Thompson§	Positive						

* Arteriolar lesions in case 2 only.

† Outer laminated and cyanophilic (PTAH) surfaces of the intra-arteriolar lesions, arterial emboli, and valvular vegetations were readily digested, as noted with fibrin.

‡ Normal elastica was red-brown (orcein) and black (Verhoeff).

§ In lesions of glomerular capillaries only.

? Doubtful or faint.

velopment of intravascular occlusion, and also precludes the possibility of their embolic nature. Such an interpretation does not refute the occurrence of superimposed platelet and fibrin thrombi upon such damaged valves and endothelial surfaces. Indeed, in many of the lesions fibrin could be identified, although it appeared quantitatively to be in lesser amount (Fig. 8). This was well demonstrated in the lesions noted within the glomerular tufts in case 1, in which fibrin also, as well as hemoglobin, could be detected. The impression was obtained that the fibrin and hemoglobin lakes were located for the most part proximal to the fibrinoid masses within the tuft, indicating the obstructive nature of this latter substance. These vascular lesions appeared morphologically and, in some respects, tinctorially similar to those noted by Bernath[17] and Sümegi and associates,[18] also in patients with carcinoma. These investigators were impressed by the affinity of the intramural material for elastic tissue dyes and Congo red as utilized in the demonstration of amyloid. However, unlike their "elastic amyloid," the occlusive and intramural substance observed in this study failed to demonstrate a significant affinity for these dyes, being similar in this respect to fibrinoid. The question whether such divergent results indicate different substances comprising the vascular lesions occasionally observed in patients with carcinoma, or merely reflect technical differences in the methods utilized, merits further investigation. It is of interest that Sümegi and associates have considered such lesions to represent the morphologic manifestations of antigen-antibody reactions and Teilum[19] has described apparently similar vascular alterations in the tissues of various diseases purportedly of a hypersensitivity nature, including lupus erythematosus. Our observations concerning the nature of the valvular lesions encountered in the patient with bronchogenic carcinoma (case 1) are in complete accord with those made previously by Allen and Sirota[20] and Angrist and Marquiss[21] concerning the nature of these lesions. The term degenerative verrucal endocardiosis, as proposed by the former investigators, appears most applicable for such lesions in the light of these findings.

The nature of the vascular lesions suggests the possibility of an immuno-allergic mechanism in their pathogenesis in which the vascular walls represent the shock site, as proposed by Beigelman[22] in other so-called collagen diseases. In addition, such mechanisms may explain some of the manifestations of the so-called generalized intravascular coagulation syndrome, notably thrombocytopenia, which were encountered in case 1. Dameshek[23] and Stefanini and Dameshek[24] have

proposed, with some experimental support, the concept that a variety of hematologic disturbances might result from immuno-allergic responses directed toward the various cellular components of the blood and/or blood vessels. These disorders include auto-immune hemolytic anemia, hemolytic anemia with thrombocytopenia, immuno-granulocytopenia, anaphylactoid purpura with and without thrombocytopenia, and thrombohemolytic thrombocytopenic purpura (Moschcowitz syndrome, thrombotic thrombocytopenic purpura). It is significant that except for the presence of hemolytic anemia the clinical features of the patient with bronchogenic carcinoma (case 1) are similar to those encountered in patients with thrombotic thrombocytopenic purpura and the vascular and valvular lesions are morphologically and tinctorially identical.[16]

Whether the thrombocytopenia observed in case 1 can be explained by an immunologic response against platelets as well as vessels cannot be established, although such an alteration would be consistent with this unified concept. The absence of thrombocytopenia in case 2 would therefore indicate the lack of a response directed against platelets. On the other hand, the absence of such changes in this patient might also reflect the importance of the deposition of fibrin and platelets upon the damaged valves and endothelial surfaces in the production of some of the clinical manifestations of the so-called intravascular coagulation syndrome. Reference to the analogy between the vascular alterations encountered in patients with carcinoma and other examples of the so-called intravascular coagulation syndrome and the generalized Shwartzman reaction has been made previously in this report. Recent studies[25,26] have revealed the fibrinoid nature of the vascular lesions encountered in the latter, and their similarities to various systemic fibrinoid diseases in man have been established.[26] It is of interest to note that the generalized Shwartzman reaction, which represents a laboratory model for conditions similar to those encountered in various infections and hypersensitivity states, is also accompanied by thrombocytopenia.[27]

Those identifying the occlusive arterial and arteriolar lesions occurring in patients with carcinoma as fibrin thrombi[8,9] have suggested their possible origin from thromboplastin, proteolytic enzymes, mucins, or products of tissue necrosis elaborated by the neoplasms. The clinical manifestations, such as purpura, thrombocytopenia, fibrinogenopenia, and prolongation of prothrombin time, have been considered to be largely dependent upon this generalized intravascular coagulation. Despite experimental evidence[28] which indicates the thrombogenic nature

of these substances, such a hypothesis appears teleologic in the light of the findings disclosed by this study. The possibility that the lesions encountered result from an immune response to such neoplastic products, particularly those of protein nature, appears more tenable from this as well as other studies, and McCombs and MacMahon[29] postulated a similar mechanism to explain the association of dermatomyositis and carcinoma.

It is not our intention to imply that all examples of the so-called intravascular coagulation syndrome cited have lesions or underlying pathogenic mechanisms which are similar to those observed in these patients with carcinoma. However, it does appear germane to note that examination of the tissues from several examples of infantile enteritis with the clinical manifestations of the so-called generalized intravascular coagulation syndrome revealed vascular and endocardial lesions entirely similar to those described in the patients comprising this report. Such findings indicate the need for similar studies of other examples of the so-called intravascular coagulation syndrome in an attempt to clarify the pathogenesis and to establish their inter-relationships, if any.

Other pathologic and clinical features encountered in the patient with bronchogenic carcinoma (case 1) are worthy of comment. The similarity of the morphologic and tinctorial features of the occlusive masses within the left coronary, splenic, and superior mesenteric arteries and of the valvular lesions establish the embolic nature of the former from this site, particularly in the absence of intramural changes in the arteries other than those attendant on early organization. This instance represents approximately the 70th reported example of coronary occlusion resulting from embolization. The occurrence of embolic phenomena with degenerative verrucal endocardiosis, although not widely appreciated, has been emphasized previously.[20,21] The morphologic appearance of the myocardial infarct can be correlated chronologically with the brief episode of shock experienced by this patient, although activation of fibrinolysin and the development of the disseminated arteriolar lesions also have been reported[9,24] as possible inciting factors for such collapse and cannot be dismissed conclusively. It is difficult to delineate the factors involved in the production of purpura in this patient. Certainly the vascular lesions with resulting escape of red blood cells as in anaphylactoid purpura, as well as the thrombocytopenia and circulating fibrinolysins which may have been present, may each have contributed to the development of this manifestation.

Examination of the venous lesions present in case 1 failed to disclose

any alterations in the veins which might predispose to thrombosis, our experience in this respect being similar to that of others.[4,5] Admittedly, the mechanisms of venous thrombosis are complex and, despite many investigations, still have not been completely defined. Yet, the possibility that venous thrombi when found in persons with various types of carcinoma might be induced by thrombogenic substances produced by these neoplasms is worthy of consideration. That these might represent a local effect augmented by other factors which are contributory to venous thrombosis of the extremities rather than a systemic alteration in the coagulation mechanism is in keeping with the normal coagulation times observed in this patient as well as others with this syndrome. Such a concept is also compatible with the information obtained from this study concerning the nature of the disseminated arterial, arteriolar, and capillary lesions noted in these patients. It might be added, in further support of the latter, that it would not appear unreasonable to expect to encounter arterial thrombi in patients with venous thrombosis more frequently if these were primarily the result of a systemic alteration of the coagulation mechanism. Yet, this has not been the experience in patients with such venous lesions whether or not they are accompanied by carcinoma.

Summary

Various tinctorial studies revealed the nature of the occlusive material observed within the lumina of small arteries, arterioles, and capillaries in two patients with carcinoma (of lung and of stomach) to be more compatible with fibrinoid than with fibrin, amyloid, collagen, or "elastic amyloid." The appearance of fibrin in such lesions appeared to be a secondary phenomenon. The material comprising the valvular vegetations was entirely similar to that observed within the small arteries and arterioles, confirming the studies of others regarding the nature of these lesions.

These results suggest the possibility that such vascular and cardiac lesions might represent an immuno-allergic response, perhaps to altered proteins elaborated by the neoplasms rather than a primary disturbance in the coagulation mechanism of the blood.

The occurrence of emboli to the coronary, splenic, and superior mesenteric arteries in one of the cases emphasizes the possibility of such sequelae from degenerative verrucal endocardiosis (indeterminate or thrombotic non-bacterial endocarditis). This represents approximately the 70th recorded instance of coronary occlusion due to embolus.

We wish to thank Dr. George Fetterman, Childrens Hospital, Pittsburgh, Pennsylvania, for the opportunity of studying the examples of infantile enteritis mentioned in this report.

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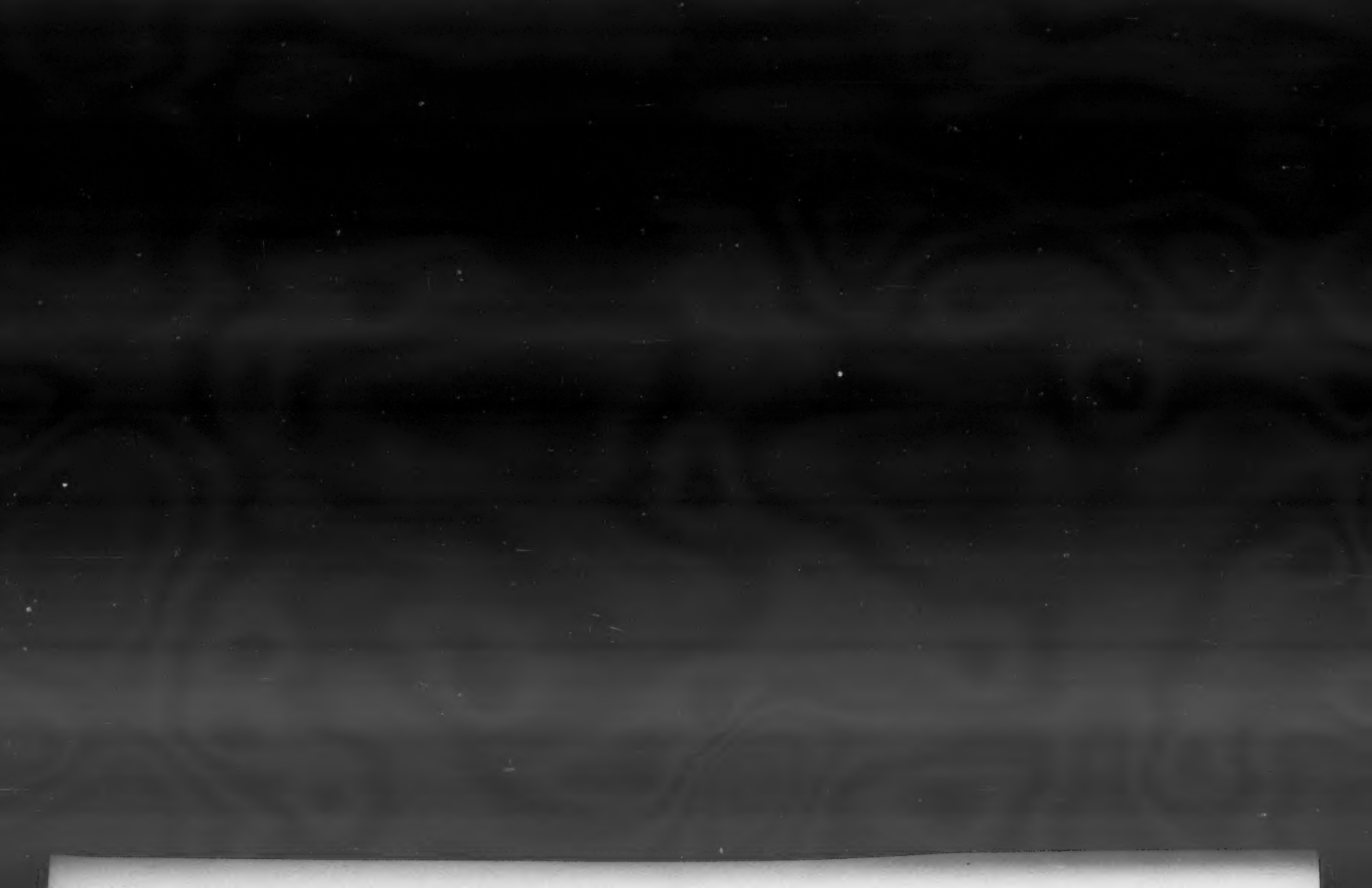
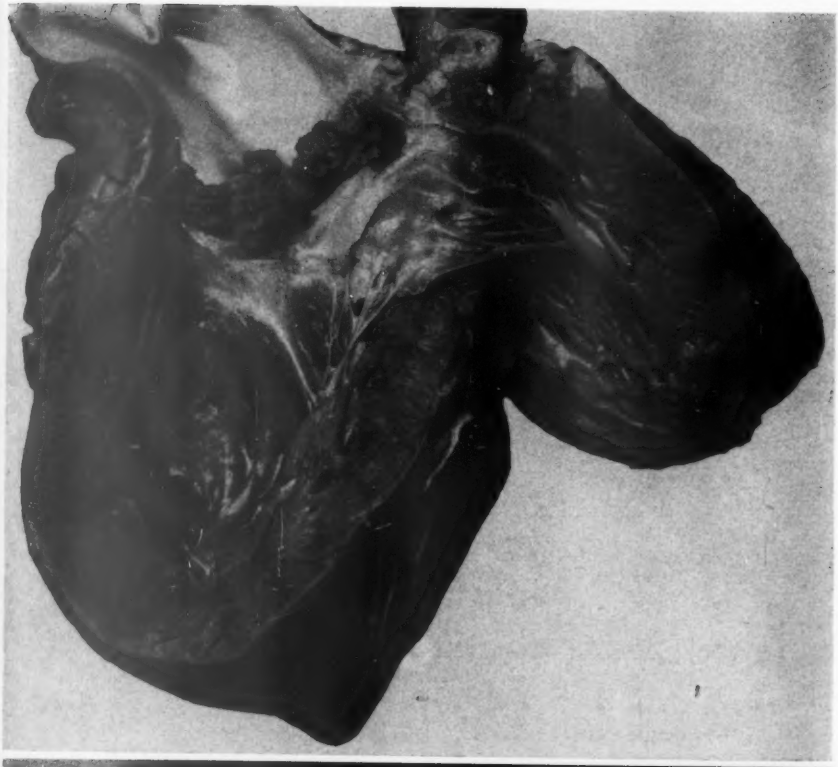
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 1. Vegetations of degenerative verrucal endocardiosis on the aortic valve. The pale area in the apex of the heart represents a zone of myocardial infarction.

FIG. 2. Case 1. Cross section of a descending branch of the left coronary artery, demonstrating embolic occlusion of the lumen.





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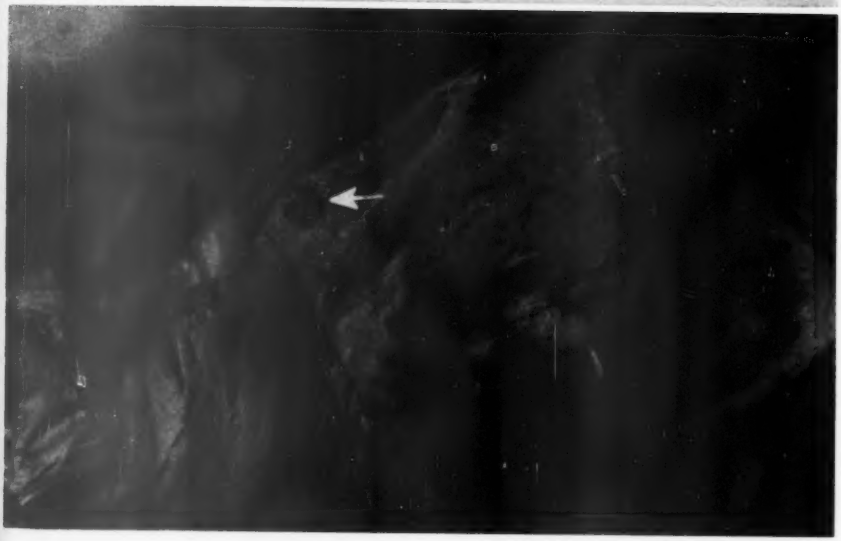

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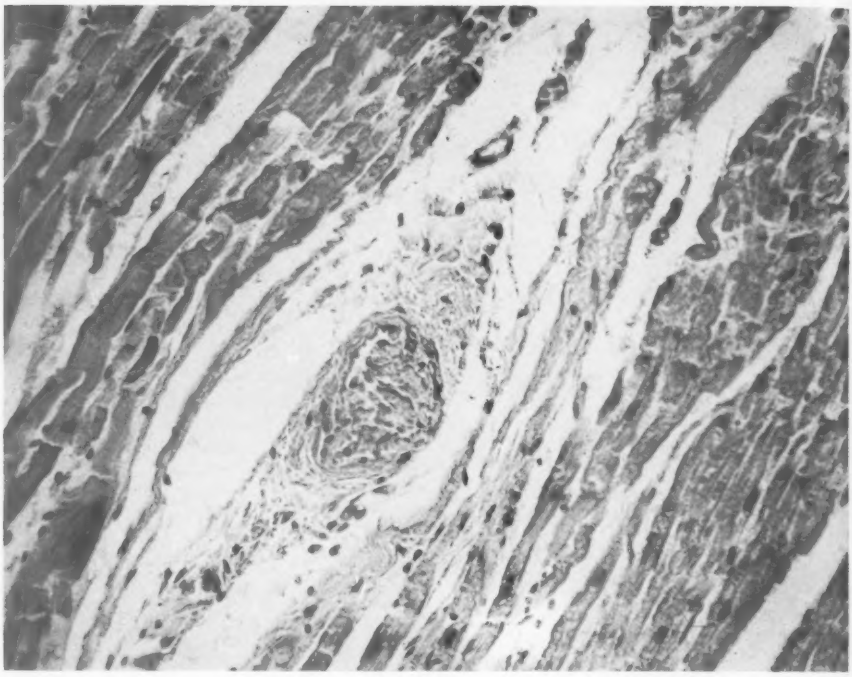
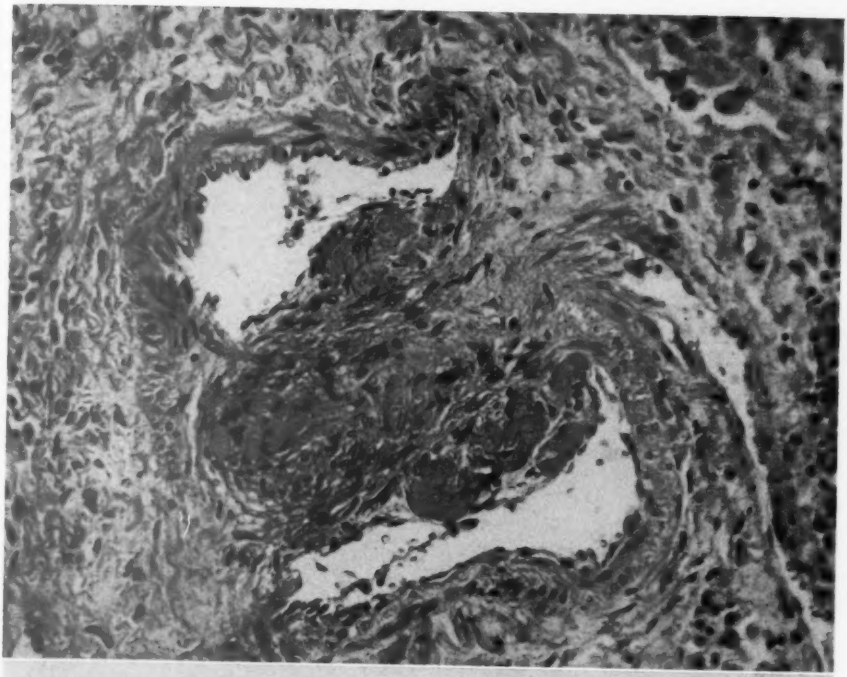


FIG. 3. Section of the myocardium, revealing luminal occlusion of an arteriole due to a fibrinoid mass. × 200.

FIG. 4. Section of the myocardium, showing fibrinoid degeneration of the wall of small arteries with beginning herniation into the lumen. The endothelium remains intact. × 230.

FIG. 5. Vegetation of the aortic valve stained by Wilder's method, demonstrating argyrophilia. × 135.



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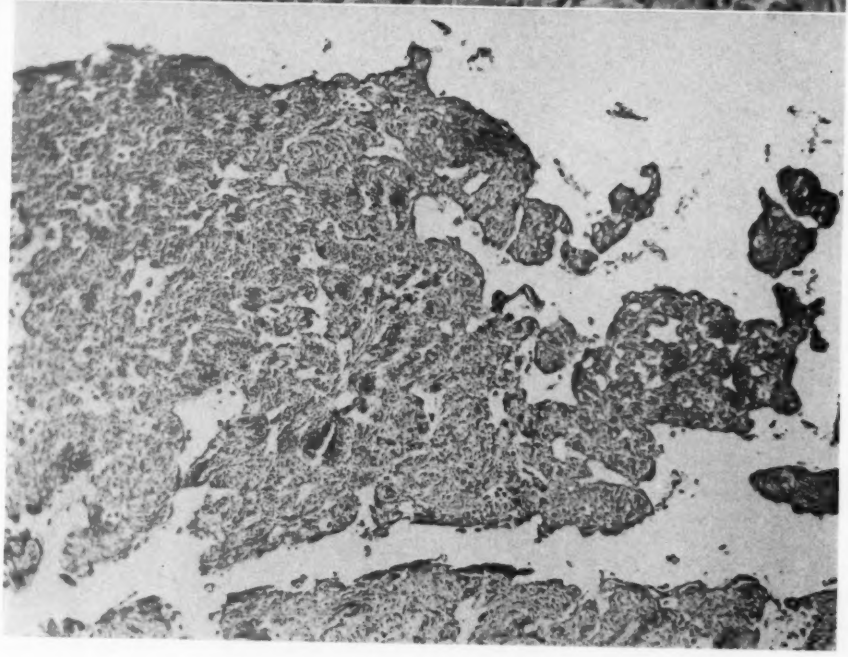

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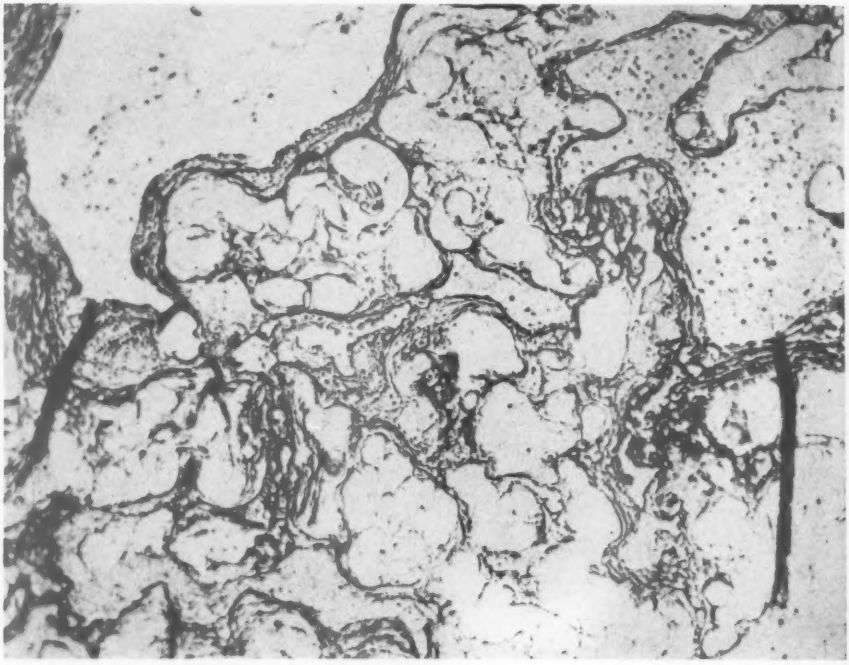
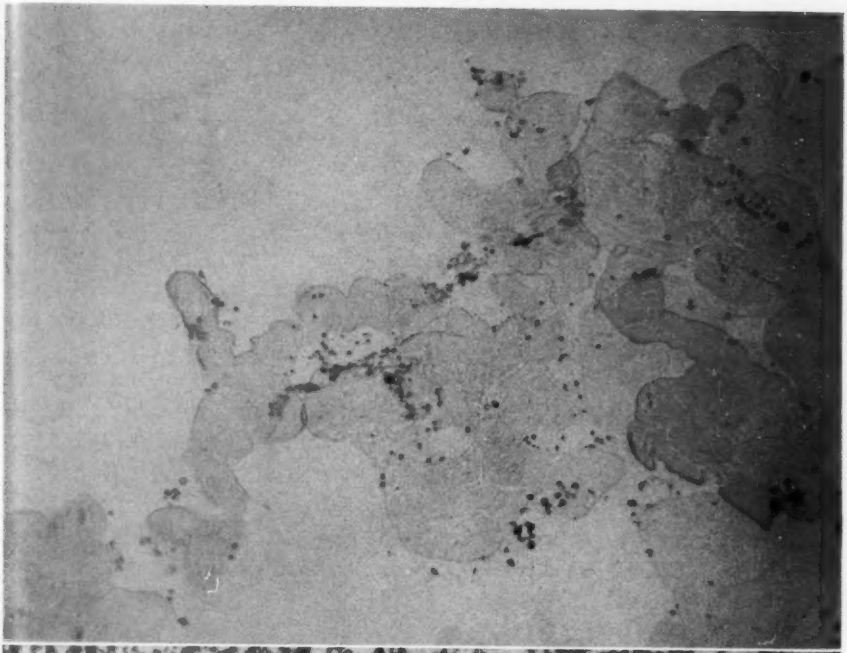


FIG. 6. Vegetation of the aortic valve stained by the phosphotungstic-acid hematoxylin (PTAH) method, revealing outer fibrillary masses (appearing black) and inner fibrinoid (appearing white). × 135.

FIG. 7. Section of aortic vegetation stained by the PTAH method after tryptic digestion. The fibrin has been removed, but the fibrinoid component persists. × 135.

FIG. 8. Section of spleen stained by the PTAH method, revealing an occlusive luminal mass comprised of two components as noted in the vegetations. The portion appearing black was cyanophilic and was digested by trypsin, whereas the clear component was orange and resisted digestion. × 215.



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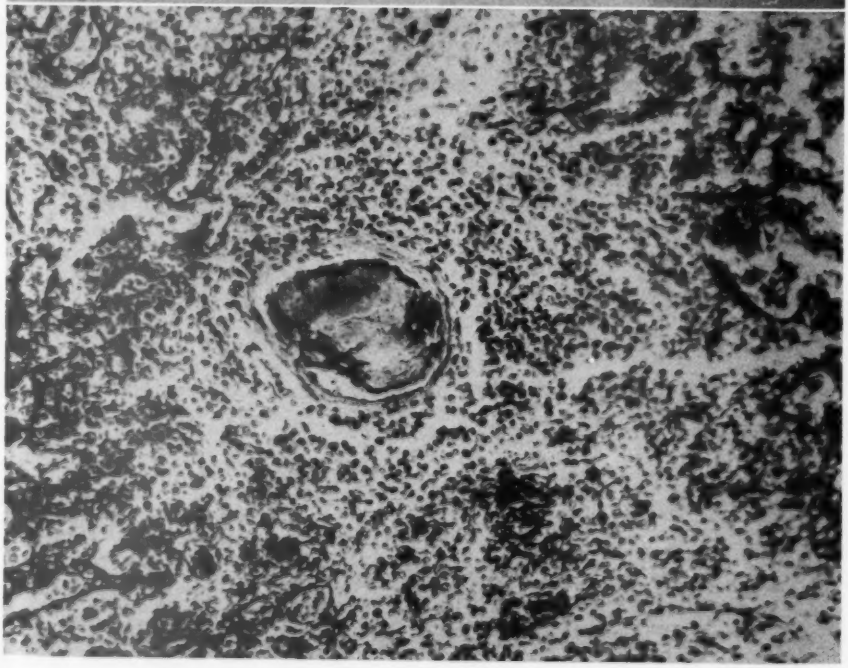

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SKIN STEROLS. XI. A DIRECT DEMONSTRATION OF FAST-ACTING STEROLS IN THE SEBACEOUS GLANDS *

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Whereas cholesterol ("Δ^5-cholestenol") is present in every animal cell, sometimes in substantial amount, the related compound Δ^7-cholestenol (lathosterol) usually occurs only in traces, if at all.[1] However, in this respect the epidermis of rats and mice differs from other tissues, chemical analyses indicating about one half of the epidermal sterols to be Δ^7-cholestenol.[2-4] The absolute amount varies with age,[5] sex,[1] and with exposure to mineral oils,[3] ultraviolet light,[4] or carcinogens.[3] Ninety per cent of the Δ^7-cholestenol in skin is found in the lipids extracted from a preparation of epidermis and sebaceous glands that has been separated from the dermis by soaking the skin in 0.33 N NH_4OH for ½ hour.[4] It is not known whether Δ^7-cholestenol is distributed uniformly throughout the epidermis or is concentrated in the skin adnexa. Before the presence of Δ^7-cholestenol in skin was known, sections stained with the standard Liebermann-Burchard method[6] revealed high concentrations of sterol in the epidermis, sebaceous glands, and dermis,[7,8] and the sterol was assumed to be cholesterol. These histochemical findings have now been re-examined by a reaction[9] that differentiates between the two sterols on the basis of the rates at which they form colors with a modified Liebermann-Burchard reagent.

Methods

Appropriate compounds known to alter the epidermal sterols were applied with a tuberculin syringe to the shaved backs of adult mice. Applications of 0.2 ml. were made on the first, third, and fifth days, and the skin was removed on the sixth day. The subcutaneous muscle was scraped from the dermis and the skins were fixed in 4 per cent formaldehyde. Representative samples of skin were dehydrated, placed in paraffin blocks, cut, and stained with hematoxylin and eosin. These sections were examined for the extent of epidermal irritation and the

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by Grant C-2177, National Institutes of Health, U.S. Public Health Service.

Received for publication, March 24, 1956.

condition of the sebaceous glands. Other samples were fixed in formalin, frozen, and cut into sections 6 to 8 μ in thickness; these were stained with Sudan III or the Liebermann-Burchard reagent without incubating the sections in ferric alum. The sites of sudanophilia were readily established in the normal and treated skins. Other sections were floated onto slides and air dried at 60°C. for 60 minutes. After drying, two to three drops of acetic anhydride-sulfuric acid (4:1, cool while mixing*) were placed on the frozen cut sections and the rate of color development observed through a microscope. To establish that cholesterol and Δ^7-cholestenol develop color at different rates of speed in tissue slices, frozen-cut sections of rat skin and of human aorta with an atherosclerotic plaque were mounted on the same slide. Repeated examinations were made on the speed of color development in specimens from several rats and human aortas. Frozen-cut sections of formalin-fixed rat intestine, liver, and brain also were examined. The presence of fast-acting sterols (Δ^7-cholestenol and 7-dehydrocholesterol) in the sebaceous glands of normal and treated mouse skin was studied to determine whether the histochemical changes parallel the alterations of Δ^7-cholestenol as determined by chemical analysis.

Experimental Results

Formalin-fixed skins from mice that had been treated with acetone solutions of various carcinogens were mounted in paraffin blocks, cut, and stained with hematoxylin and eosin. Other formalin-fixed tissues were cut in the frozen state and then treated with Sudan III and the concentrated Liebermann-Burchard reagent. The principal microscopic changes observed in these sections are shown in Table I.

Observations Made on Sections Stained with Hematoxylin and Eosin. The normal mouse epidermis was 3 to 5 cells wide; the sebaceous glands were well defined structures in the upper dermis and were attached to the epidermis by the hair follicles. Methylcholanthrene, benz(α)pyrene, and croton oil induced hyperplasia of the epidermis (Table I, nos. 2, 3, 6, and 7). In every instance this hyperplasia was associated also with either leukocytic infiltration (nos. 6 and 7), or edema and leukocytic infiltration (nos. 2 and 3). Octadecane and dodecene produced epidermal hyperplasia without inflammation or edema (nos. 8 and 9). Only methylcholanthrene in acetone destroyed the sebaceous glands in 6 days (no. 2). The other chemicals exerted

* Greater concentrations of sulfuric acid also produce a bluish color in sebaceous glands but the tissues disintegrate rapidly; at lesser concentrations of sulfuric acid color formation in the sebaceous glands was inconstant.

TABLE I

Effects of Certain Hydrocarbons on Mouse Skin as Observed with Various Techniques

	Compound applied	Hematoxylin and eosin			Sudan III		Liebermann–Burchard	Chemical analysis*	
		Epidermal thickness	Leukocytic infiltration	Sebaceous glands	Follicular channel	Sebaceous glands	Sebaceous glands	Epidermal Δ^7-cholestenol/cm.^{2}	Epidermal cholesterol/cm.^{2}
								μg.	μg.
1	None	3–4 cells	–	Normal	+	+++	+++	52.2±9.2	51.6±11.4
2	0.2% Methylcholanthrene in acetone	5–6 cells	+ and edema	Absent	–	–	–	12.5	96.5
3	0.2% Benz(α)pyrene in acetone	7–10 cells	+ and edema	Atrophic	+	+	+	30.5	108.5
4	0.2% Dibenzanthracene in acetone	5–6 cells	+	Atrophic	+	+	+	39.0	78.0
5	0.3% Benzanthracene in acetone	2–3 cells	–	Normal	+	+++	+++	66.0	104.0
6	4.0% Croton oil in acetone	5–8 cells	+	Atrophic	+	+	+	62.5	180.0
7	0.5% Methylcholanthrene in USP mineral oil	6–7 cells	+	Slightly swollen	–	++	++	23.0	62.0
8	Octadecane	7–8 cells	–	Slightly swollen	+	+++	+++	47.0	115.0
9	Dodecene	5–6 cells	–	Slightly swollen	+	+++	+++	60.6	129.5

* These values were obtained from the analysis of the extracted lipids from the treated skins. The procedures and discussion are included in reference 10.

questionable morphologic effects on the sebaceous glands (nos. 3 to 9).

Observations Made on Sections Stained with Sudan III. Examination of the frozen-cut sections which were stained with Sudan III revealed intense sudanophilia in sebaceous glands from normal mice and test animals treated with the non-carcinogens, benzanthracene, octadecane, and dodecene (Table I, nos. 1, 5, 8, and 9). Even though the structure of the sebaceous glands was not significantly altered in the hematoxylin and eosin sections, much less sudanophilia was evident in the skins of some mice (nos. 3, 4, and 6). Sudanophilia of the sebaceous glands was not evident in the skins of mice treated with methylcholanthrene in acetone (no. 2).

Observations Made on Sections Stained with the Liebermann-Burchard Method. After two to three drops of Liebermann-Burchard reagent were placed on a dried section of frozen-cut normal mouse skin, microscopic observation disclosed a purple color in sebaceous glands within 2 to 3 minutes, which faded after 5 minutes (Table I, no. 1). In normal rat skins prepared similarly and treated with the Liebermann-Burchard reagent, the purple color was produced in the sebaceous glands within 2 minutes; in those glands with the least intensity of color at 2 minutes, fading to gray or light brown occurred in 5 minutes, whereas the more intensely colored sebaceous glands retained the purple color in the neck of the gland and in the follicular shaft for as long as 12 minutes. This variation in fading time is believed to be a concentration effect. In the skins of mice treated with methylcholanthrene in acetone, the characteristic color reaction of the fast-acting sterols was absent (no. 2). On histologic observation, less fast-acting sterol was observed in the sebaceous glands from treated skins in nos. 3, 4, and 6; whereas, in skins from other test animals color development appeared normal (nos. 5, 8, and 9). The application of a 0.5 per cent solution of methylcholanthrene in USP mineral oil did not destroy the sebaceous glands within the 6-day period (no. 7), but the glands were swollen, and they contained decreased amounts of sudanophilic material and fast-acting sterol. The Liebermann-Burchard reaction in the sebaceous glands therefore closely parallels the sudanophilia which was observed in these structures.

Chemical Analysis[10] *of Mouse Skins Painted with Solutions of the Carcinogens.* Correlation between chemical analyses and histochemical observations showed a decrease in fast-acting sterols in proportion to the increasing potency of the applied carcinogen. At the same time there was an increase in the amount of cholesterol corresponding to the degree of epidermal hyperplasia produced (nos. 2, 4, and 6).

Analysis of epidermis treated with 0.5 per cent methylcholanthrene in USP mineral oil showed that slightly more Δ^7-cholestenol remained after application in a viscous oil than after its application in acetone (nos. 2 and 7). The chemical analysis of many samples of skin made hyperplastic with hydrocarbons like dodecene and octadecane showed the fast-acting sterol content to remain normal and the amount of cholesterol to increase with increasing thickness of the epidermis (nos. 8 and 9). Table I shows a good correlation between chemical analyses and the histochemical demonstration of fast-acting sterol except in the skin of a mouse treated with 4 per cent croton oil (no. 6) in which the hyperplasia was extreme.

Comparison of Color Formed in Sebaceous Glands with That Which Develops in an Atheromatous Plaque and Other Tissues. In order to compare the staining reactions of cholesterol and Δ^7-cholestenol in tissues, frozen-cut sections of rat skin and atheromatous aorta were placed in parallel on the same slide. After the Liebermann-Burchard reagent was applied, the reactions were observed and photographed (Fig. 1). A purple color developed in normal sebaceous glands within 2 to 3 minutes (Fig. 2), which gradually decreased and changed to gray or brown by 12 minutes (Fig. 4). Cholesterol in the aortic plaque, however, required more than 5 minutes to attain an intense blue-green color (Figs. 3 and 4) which persisted for more than 30 minutes. In contrast to the color development in the sebaceous glands, the cholesterol in the lipid plaque in the aorta dissolved and floated in the reagent. The application of the concentrated Liebermann-Burchard reagent (4:1) to frozen sections of rat small intestine, liver, and brain did not reveal the presence of fast-acting blue color characteristic of Δ^7-cholestenol. Only the brain slices showed the delayed blue-green color of cholesterol, which dissolved and floated in the reagent.

Kodachrome Photographs. Photographs of these changes, when projected, revealed a zone of bluish coloration at the edges of fat cells in the dermis. This blue color was not seen on direct observation and was present when the skin section was exposed to acetic anhydride alone. This color, therefore, was attributed to some effect other than the characteristic sterol reaction, which does not develop with acetic anhydride itself. Microscopic observation of 0.1 mg. of crystalline or solid Δ^7-cholestenol reacting with the concentrated Liebermann-Burchard reagent (4:1) showed an immediate formation of a purple color which became blue after 1½ minutes and changed to gray by 12 minutes. Under similar circumstances pure cholesterol developed after 1½ minutes a blue-green color which persisted for several hours.

Discussion

The Liebermann-Burchard reagent (sulfuric acid in acetic anhydride) has long been known to form a green chromophore with cholesterol. Other sterols also form colors with this reagent, but the rates of formation and fading vary considerably from one sterol to another.[9] With glacial acetic acid as the solvent and a relatively "dilute" form of the reagent (20 parts acetic anhydride and one part sulfuric acid) "fast-acting sterols" such as Δ^7-cholestenol and 7-dehydrocholesterol form a maximum color at 90 seconds with marked fading thereafter, while cholesterol produces no color at 90 seconds but reaches its maximum optical density at 38 minutes.[9] These differences between sterols persist whether the sterol is esterified or free, and they also persist when the solvent, the concentration of sulfuric acid, or the temperature is varied, although these latter changes affect the absolute times at which maximum color is produced.

Earlier methods used to stain for cholesterol in the skin (developed before the presence of Δ^7-cholestenol was known) showed the sterol to be distributed throughout the skin and in the sebaceous glands. The green color of the sparsely distributed sterol was accentuated by incubating the frozen sections for 72 hours in ferric alum before staining with acetic acid-sulfuric acid (4:1).[6] The technique used in the present study does not stain cholesterol that is diffused throughout tissues but only when it is highly concentrated as, e.g., in aortic plaques or the brain. In these latter locations, however, color forms at a slower rate than with the fast-acting sterols of the sebaceous glands, and the shade of color also is different.

It is not known whether the small fraction (25 per cent) of esterified cholesterol in the extracted lipids of epidermis exists also in the sebaceous glands; however, this ester does not decrease with the destruction of sebaceous glands by methylcholanthrene in acetone.[11] The possibility that some of the Δ^7-cholestenol is distributed throughout the cells of the epidermis appears unlikely in view of the unvarying concentration of this extracted sterol per square centimeter of epidermis even in extreme epidermal hyperplasia, while in similar circumstances cholesterol may increase four-fold.[10]

A number of additional observations suggest that Δ^7-cholestenol may be concentrated in the sebaceous glands. The rapid disappearance of fast-acting sterol from the extracted epidermal lipids following the application of methylcholanthrene in acetone[3] parallels the histologic destruction of the sebaceous glands under the influence of this carcinogen.[12] Suskind[8] and Montagna and Norback[7] have shown histologi-

cally that the Liebermann-Burchard-positive sterols in the sebaceous glands were esterified; the Δ^7-cholestenol in the extracted lipids of rodent skin is almost completely esterified whereas most of the cholesterol in skin occurs in the free form.[4] Sebaceous glands are particularly abundant in the skin of hairless mice, and the extracted lipids of such skin contain substantially more Δ^7-cholestenol than of ordinary mouse skin.[13] Further circumstantial evidence is the observation that Δ^7-cholestenol is abundant in sebaceous cysts,[14] and in sebum.[15] Conversely, the skin of young mice is relatively deficient in extracted Δ^7-cholestenol[5] prior to the full development of the sebaceous glands.

The significance of the specific location of Δ^7-cholestenol in the sebaceous glands is not yet understood. However, its co-existence with pro-vitamin D_3 (7-dehydrocholesterol) suggests a function related to this physiologically important compound.[1]

Summary

The presence of Δ^7-sterols in the sebaceous glands of mice and rats has been demonstrated by staining frozen sections with concentrated Liebermann-Burchard reagent. Under these conditions an immediate but transient blue color appeared in the sebaceous glands, while the blue-green color due to cholesterol in an aortic atherosclerotic plaque formed at a much slower rate and persisted for a longer time. These differences in color formation parallel known differences between cholesterol and Δ^7-cholestenol (or 7-dehydrocholesterol) as pure crystals or in solution.

Procedures known to reduce the Δ^7-cholestenol content and increase the amount of cholesterol (application of carcinogens) resulted in the degeneration or destruction of sebaceous glands and a decrease in sudanophilic material in the epidermis. Such epidermis also showed a decrease in the Liebermann-Burchard fast-acting chromogen in frozen sections.

Procedures that increased the thickness of the epidermis and the amount of cholesterol without causing a change in the amount of Δ^7-cholestenol (application of long chain alkanes and alkenes) produced slightly hypertrophied, Sudan-stainable sebaceous glands. These glands gave the normal fast-acting color of sebaceous glands upon addition of the concentrated Liebermann-Burchard reagent.

We are indebted to Homer Montague for his cooperation in the preparation of photographs.

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LEGENDS FOR FIGURES

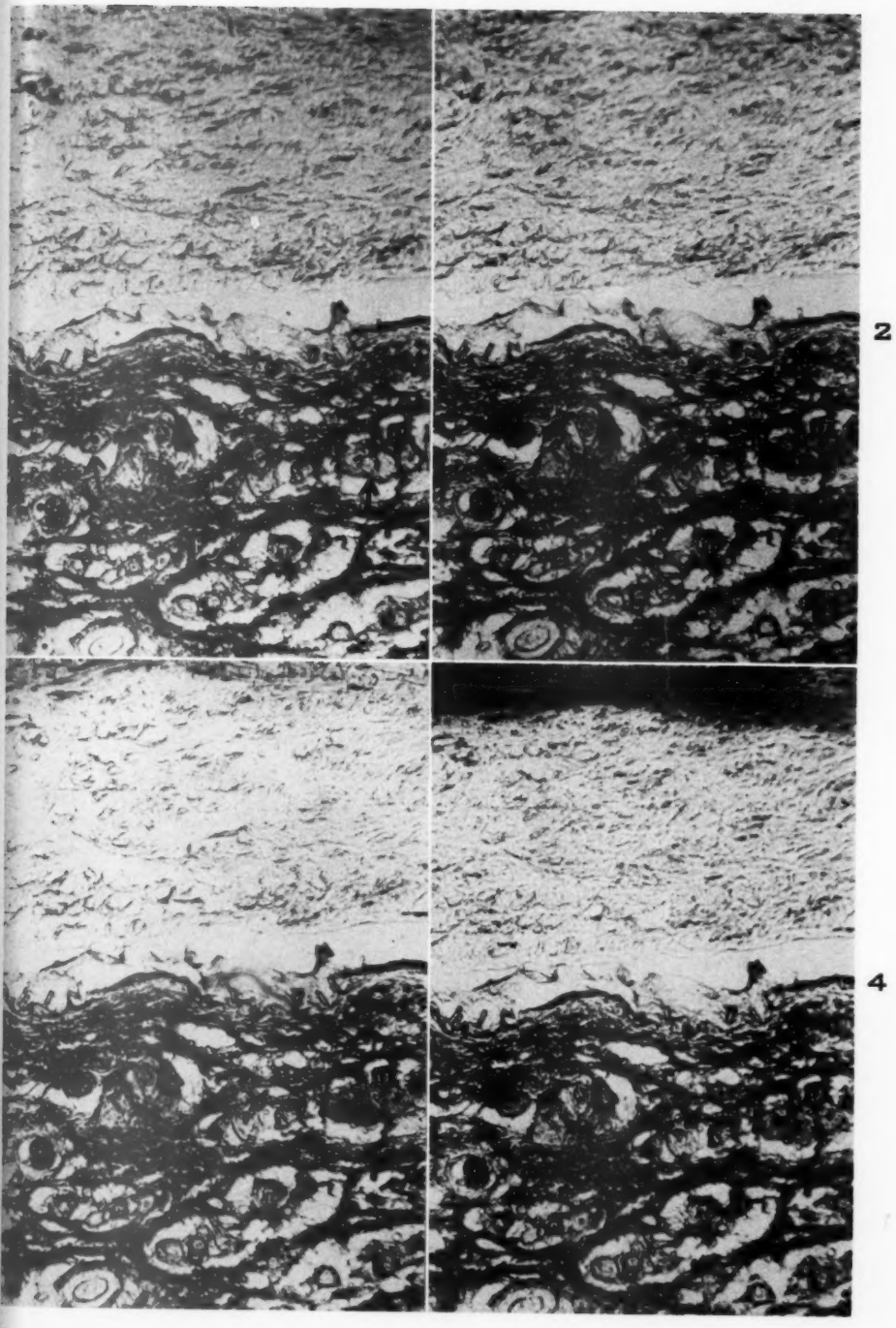
Photographs numbered 1 to 4 are of the same field taken at different time intervals following the addition of concentrated Liebermann-Burchard (4:1) reagent. A lipid plaque from a human aorta is above while rat skin with two sebaceous glands identified by arrows is at the bottom (Fig. 1). The photographs were taken at a magnification of 40 × with a yellow filter interposed over the light source.

FIG. 1. (At 18 seconds.) At this time color is not apparent in the sebaceous glands or the aorta. The dark spheroids at the upper left are due to air bubbles.

FIG. 2. (At 2 minutes and 40 seconds.) A purple-blue color has developed in the sebaceous glands.

FIG. 3. (At 4 minutes and 15 seconds.) The purple-blue color due to Δ^7-cholestenol usually fades after 3 or 4 minutes. The color in the sebaceous glands by this time has changed to a gray or light brown. The blue-green color of cholesterol begins to appear in the intima of the aorta.

FIG. 4. (At 7 minutes and 55 seconds.) The sebaceous glands retain their light brown color, whereas the blue-green in the intima becomes more intense.



MYOCARDIAL REGENERATION IN YOUNG RATS*

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The problem of the regenerative capacity of heart muscle has been under discussion for the last hundred years; many papers have been written, but little has been contributed to challenge the conclusion that the myocardium takes a minimal share in the healing of cardiac injuries.

Histologic studies of the hearts of those dying of diphtheria or with cardiac hypertrophy, chiefly children,[1-3] have suggested that there may be true regeneration of cardiac muscle fibers, both by longitudinal splitting of the fibers and by mitotic division. This suggestion, the postulate that in infants regeneration of the myocardium may be possible due to persisting physiologic development, and the fact that the literature contains no data concerning the experimental production of myocardial lesions in newborn animals, have been the factors in my decision to explore the possibility of myocardial regeneration in newborn rats.

REVIEW OF THE LITERATURE

There have been several reviews of the rather extensive literature about hyperplasia and regeneration of the myocardium, of which the more important were by Goldemberg[4] (1886), Goldzieher and Makai[5] (1912), Karsner and Dwyer[6] (1916), Karsner, Saphir, and Todd[7] (1925), MacMahon[8] (1937), and Ring[8] (1950).

Goldemberg,[4] in 1886, brought out the first review of the literature since 1845, and dealt mainly with the problem of hypertrophy of the myocardium. He discussed the earlier papers of Vogel,[9] von Kölliker,[10] Förster,[11] Lebert,[12] Hyrtl,[13] von Rokitansky,[14] Heschl,[15] Hepp,[16] Robin,[17] Wedl,[18] Becquerel,[19] Friederich,[20] Wilks and Moxon,[21] and Rindfleisch.[22] He pointed out that the controversy concerning myocardial hypertrophy had become well established. Some believed that hypertrophy of the heart is due to simple enlargement of the individual muscle fibers, while others accepted, and described, true hyperplasia and even longitudinal splitting of the myocardial fibers (Wilks and Moxon[21] and Rindfleisch[22]).

* Submitted in partial fulfillment of the requirements for the degree of Master of Science in Pathology, University of Michigan.
Received for publication, February 27, 1956.
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Zielonko,[23] in 1874, under the supervision of Virchow, did the first experimental work in this field. He studied hearts of rabbits and frogs and came to the conclusion that hypertrophy is due to the enlargement of individual muscle fibers, and probably also to cellular hyperplasia. Goldemberg[4] concluded as a result of his work that hypertrophy of the heart is due to increase in size of individual muscle fibers rather than to true hyperplasia. Karsner *et al.*,[7] in 1925, after measuring cardiac muscle fibers of normal, hypertrophic, and atrophic hearts, concluded that cardiac enlargement is due to hypertrophy of individual muscle fibers without increase in their number. MacMahon,[3] in 1937, studied 3 cases of cardiac hypertrophy in infants (6, 12, and 20 months of age), due to different causes. He found numerous mitotic figures.

The regenerative capacity of the myocardium has been discussed controversially also from the point of view of the healing of toxic myocarditis, myocardial infarction, and wounds of the heart. Saltykow,[24] in 1905, published a work on diffuse myocarditis in which he stated that the muscular elements themselves play an important part in healing by forming new fibers. Heller,[1] in 1913, described the regeneration of heart muscle fibers in diphtheritic myocarditis by their longitudinal splitting. Warthin,[2] in 1924, studied 16 cases of diphtheritic myocarditis and found evidence of signs of regeneration in 3 of them. His description was as follows:

> Near the necrotic or degenerated portions of the heart muscle, the nuclei of the muscle show a great variety in size and form. They increase in length and show many evidences of longitudinal splitting in every possible stage of such a division. The living muscle substance bordering on the injured area also undergoes a longitudinal splitting into muscle bands containing nuclei; these bands grow into the perimysial tubes, filling these up, replacing the cell detritus, and connecting with the living muscle on the other side of the defect. Muscle bands without nuclei in their substance but accompanied by myoplastic nuclei also extend into the tubes occupied by the dead muscle substance. These bands lie at the periphery of the tube, and in some cases appear to form a hollow cylinder enclosing the remains of the dead muscle substance. . . . We have seen all of the appearances illustrated by Heller in his article on the regeneration of the heart muscle. The defects in the muscle caused by hyaline necrosis may ultimately be bridged by a number of new muscle bands apparently uniting with the living muscle on the other side of the defect. The sharp delimitation of the muscle in perimysial or sarcolemmar tubes was shown in our cases as in those of Anitschkow and Heller. These appearances, as Heller has pointed out, seem contradictory to the present accepted view that the heart muscle has no true sarcolemma. There is a close similarity between the process of regeneration of the heart muscle and that of the peripheral nerve trunks.

MacMahon,[3] in 1937, studied the heart of a child 6 years old, who died several days after onset of diphtheria, and he likewise described numerous mitotic figures in myocardial fibers which were considered

by him to be evidence of true myocardial regeneration. His conclusions were:

> Evidence is presented in the form of mitotic division of the nuclei of heart muscle fibers to indicate first, that in cardiac hypertrophy of infants a proliferation of heart muscle fibers can take place, and secondly, that in severe myocardial injury in children, regeneration of myocardial elements can occur.

Mallory, White, and Salcedo-Salgar,[25] in 1939, published a very interesting work on the speed of healing of myocardial infarction, based on the study of 72 human cases. They described the pathologic changes at different periods and compared the rate of healing in their cases with that seen in experimental infarctions. They did not mention regeneration of heart muscle fibers.

King,[26] in 1941, described changes in the muscle fibers around recent wounds in human hearts, consisting of proliferation of nuclei, mitotic figures, and outgrowth of protoplasmic masses. He wrote: "From an examination of heart wounds, the writer is convinced that some regeneration of muscle takes place." This same author, referring to the changes in myocardial infarction, stated: "Observations which strongly suggest regenerative or hyperplastic growth of muscle cells are to be made in some cases of coronary occlusion."

In the experimental field much has been done by inflicting cardiac injuries of different types, such as experimental infarction by ligation of branches of the coronary arteries, traumatic lesions of the myocardium by blows, application of heated instruments, diathermy needles, stab wounds, and experimental myocarditis produced by the intravenous injection of sparteine and adrenalin. The paper by Cohnheim and von Schultess-Rechberg[27] in 1881 was one of the first to discuss the pathologic anatomy of experimental myocardial infarction in dogs. Kolster's publication[28] (1893) was based on experimental myocardial infarction in 11 dogs; he described mitotic figures in heart muscle fibers, connective tissue, and adventitia of blood vessels at the periphery of the necrotic areas, but he concluded that no true regeneration of the myocardium occurred. Fleisher and Loeb,[29-31] in 1909 and 1910, made a series of studies of experimental myocarditis in rabbits. They found that small doses of sparteine (0.012 gm. per kg. of body weight), or caffeine (0.025 gm. per kg.), followed by the intravenous injection of a small quantity of adrenalin (0.2 ml. of a 1 to 1,000 solution), produced microscopic changes in the hearts of rabbits, located chiefly at the base of the left ventricle. They described the microscopic changes of the myocardium at different periods after injection and, although they did not find actual signs of regeneration of heart muscle

fibers, they wrote: "It appears that the myocarditic lesion actually heals and that in many cases the repair is not due to the replacement of the injured parenchyma by fibrous tissue, but by a recovery of certain muscle cells." Christian, Smith, and Walker,[32] in 1911, published a paper concerning the experimental production of cardiorenal disease in rabbits; they repeated Fleisher and Loeb's experiments and described the pathologic lesions in heart, kidney, and liver. No regeneration of the heart muscle fibers was found.

Goldzieher and Makai,[5] in 1912, as a result of their experiments and review of the literature concerning myocardial regeneration, came to the opinion that myocardial lesions probably are repaired only by connective tissue scars. They believed that the regenerative proliferation of muscle fibers, if it occurs at all, is limited to a very slight depth at the edges of a wound. Karsner and Dwyer,[6] in 1916, described the findings in experimental infarction in 13 dogs, and gave an account of the histologic changes. They did not find evidence of myocardial regeneration. Moritz and Atkins,[33] in 1938, produced cardiac contusions in 32 adult dogs by opening the thorax and striking the heart one or more forcible blows. The animals were killed at varying intervals up to 6 months. The degeneration of muscle cells due to the trauma was described, but regeneration was not found.

In 1947 Harrison[34] produced myocardial lesions in 6 rabbits by applying a diathermy needle for 10 seconds, thereby causing an area of necrosis 3 to 4 mm. wide. The animals were killed 24 hours and 3, 5, 7, 9, and 14 days after the operation. He described the changes produced by the injury but there was no regeneration. Walls,[35] in 1949, induced lesions in the lower third of the right ventricle of rabbits' hearts by the application of the head of a red-hot nail. The animals were allowed to survive for periods ranging from 3 days to 1 month and the injured areas were then studied for evidence of regeneration of the muscle fibers, but none was found. He thought that the injury was too severe, so that the reaction to it did not allow myocardial regeneration.

Ring,[8] in 1950, using rabbits and cats, 35 animals in all, produced myocardial infarction by ligation of the left coronary artery. Biopsy specimens taken 2 days to 2 months after the operation were studied for evidences of regeneration of heart muscle fibers. He observed interesting alterations at the ends of the surviving muscle fibers, especially during the period of 7 to 9 days after infarction. These consisted in loss of transverse striation, accentuation of longitudinal striation, and exaggeration of basophilism. At the same time the nuclei were

arranged in pairs and occasional mitotic figures were seen. There was expansion of the ends of muscle fibers, which sent out short processes into the surrounding tissue. These changes were interpreted as early regeneration, which did not proceed beyond the budding stage. He suggested that this was a frustrated attempt at regeneration, probably due to the absence of a sarcolemmal framework along which the new fibers could grow.

Studitskii,[36] in 1954, transplanted fragments of myocardium of young chickens and rabbits into previously made spaces in voluntary muscle of other animals of the same species, and described the changes in the graft at different periods after the operation. The most conspicuous finding was the formation of myoblasts from the heart muscle fibers of the implant. Myoblasts appeared between the fifth and seventh days after transplantation, in the form of cells of two different types, spherical and spindle-shaped. The spherical cells contained one or two nuclei with large nucleoli, and the cytoplasm was coarsely vacuolated. The nuclei of the spindle-shaped cells were oval or elongated and possessed large nucleoli, and the cytoplasm contained numerous small vacuoles parallel to the long axis of the cells, as well as elongated fibrillar structures. Numerous mitotic figures were present in these spindle-shaped cells. Later a mass of spindle cells was formed; the individual cells increased in size and contained numerous fibers which could be seen distinctly between the vacuoles. These cells were able to contract and Studitskii believed that they were actually myoblasts.

Other references to the experimental production of cardiac lesions have been omitted because they do not deal with the problem of myocardial regeneration.

From a review of the literature, the preponderant opinion seems to be that true regeneration of the myocardium does not occur in adults, but that its occurrence in infants is probable.

Material and Methods

The general experimental procedure was to expose the heart of a newborn white rat under general anesthesia, inflict a small burn, repair the incision, and allow the animal to live for a certain period. It was then killed and the reparative processes in the myocardium evaluated.

Eighty rats were used in this study, of which 58 survived for periods which made them suitable for histologic examination. Twenty-two died, their deaths being attributable mainly to the anesthesia, which, in the early stages of the experiment, had not been assayed correctly.

Some were killed by their mothers after being returned to the cages. The hearts of 8 animals which died under anesthesia before the myocardium was burned were used as controls, especially in respect to the presence of mitotic figures in myocardial fibers. The age of the animals at the time of operation was between 4 and 7 days. They were anesthetized with nembutal intraperitoneally. Under sterile precautions a small, left, parasternal incision was made through the skin, the muscles were dissected, and the 6th costochondral junction was excised. Though this small hole the heart was touched for 1 second with a wire 1 mm. in diameter which had been heated to a red heat. This small burn usually was made at the apex of the heart or on the lower third of the anterior surface of the left ventricle, the diameter and depth being very uniform in most instances (Figs. 1 and 2). The pericardium is so thin that it was easily perforated by the heated wire. The wound was closed with a single stitch at the muscular level and the skin edges were kept in apposition by a thin film of celloidin. Tracheotomy or tracheal intubation was not necessary. The pneumothorax was not aspirated. Following the operation, the animals were kept warm, and after recovery from anesthesia (in 3 to 4 hours) were returned to the cage with the mother rat. Beginning 12 hours after the operation, they were sacrificed in pairs, in an ether chamber, at daily intervals until the 15th day; then a pair was sacrificed every 5 days until the 75th day.

Necropsy was performed immediately after death and the heart was fixed in 10 per cent formalin. A block including the lesion was embedded in paraffin, and four to twelve serial sections were cut from each block. Half of them were stained with hematoxylin and eosin and half by Masson's trichrome method. In 16 cases additional sections were stained by von Kossa's method to determine the presence of calcium deposits in the lesions. All preparations were examined microscopically and described; search for mitotic figures was made with an oil immersion objective.

Results

The evolution of the lesion was followed day by day until the 15th day, and then every 5 days until the 75th day. For each period, two hearts were studied and for several intervals a third was added in order to resolve some doubts in the interpretation of the microscopic findings.

Since the gross findings added but little of interest and were very constant, because of the uniformity of the lesions, detailed gross descriptions will not be made in every case.

12 Hours after Injury (Rats 1 and 49). Grossly, after 12 hours, the lesion consisted of a 1 mm. circular, grayish defect covered by fibrinous exudate. Microscopically, coagulation necrosis, vasodilatation, congestion, margination of leukocytes, hemorrhages by diapedesis, interstitial edema, and a few polymorphonuclear leukocytes in the neighborhood of the lesion were noted. In myocardial fibers beneath and around the lesion, granular and vacuolar degeneration was present, and still deeper cloudy swelling of variable degrees was seen. On the surface there were a few threads of fibrin.

24 Hours after Injury (Rats 23, 24, and 42). After 24 hours, the necrotic tissues were infiltrated by polymorphonuclear leukocytes, a few lymphocytes, and macrophages. The myocardial fibers at the border of the lesion showed marked granular and vacuolar degeneration of the sarcoplasm, which was beginning to be removed by phagocytes, leaving empty tubes. The nuclei of these fibers showed pyknosis and karyolysis in various stages, the severity of these changes decreasing in the deeper portions. Interstitial edema, congestion, hemorrhages by diapedesis, and some leukocytic infiltration were present in the neighborhood of the lesion. In the deeper myocardial fibers it was possible to identify several mitotic figures, an observation which was confirmed by staining with Masson's trichrome method, and examining under the oil immersion objective. The interstitial connective tissue around the lesion showed several mitotic figures. On the surface there was fibrinous exudate, and fibrinous adhesions to the lung were present in rat 24.

2 Days after Injury (Rats 35 and 36). After 2 days the necrotic tissues had been partially removed leaving a considerable defect in the heart wall (Fig. 1). The area of the lesion was infiltrated by polymorphonuclear leukocytes, lymphocytes, and abundant macrophages loaded with cellular detritus; the extravasated erythrocytes were becoming hemolyzed. The interstitial connective tissue was beginning to proliferate and with Masson's trichrome stain chains of fibroblasts could be seen advancing from the edges of the defect into the remaining necrotic tissues. At the same time, angioblastic proliferation was beginning. The surviving myocardial fibers at the border of the defect showed variable degrees of granular and vacuolar degeneration, depending on their proximity to the burned area. In the fibers closest to the lesion the degenerated sarcoplasm had been removed already, leaving empty tubes. In deeper portions the muscle nuclei showed great activity, represented by all phases of mitotic division, and in such fibers there was loss of striations and the sarcoplasm appeared finely granular (Fig. 17).

3 Days after Injury (Rats 3 and 38). The lesion after 3 days was similar to that seen on the preceding day. Macrophages were more abundant and the proliferation of the interstitial connective tissue at the borders of the defect was more conspicuous, with formation of a network which was advancing into the defect. Mitotic figures were abundant in the deeper myocardial fibers and in some of them columns of two or three nuclei were seen. Young connective tissue adhesions to the costal wall had formed already in rat 38. In the adhesions there was active angioblastic proliferation with the formation of small capillaries. In this animal, also, there were small deposits of lime salts in necrotic myocardial fibers at the edges of the lesion.

4 Days after Injury (Rats 25 and 26). After 4 days there had been progress in the removal of the necrotic tissues in which there were many macrophages. Proliferation of the interstitial connective and angioblastic tissues had increased. The degenerated muscle fibers at the borders showed heavy calcareous deposits, proved to be calcium phosphate by von Kossa's reaction. In the deeper zones the myocardial fibers still showed varying degrees of granular and vacuolar degeneration, and there were macrophages and a few polymorphonuclear leukocytes and

lymphocytes. Still deeper, the myocardial fibers showed many mitotic figures (Fig. 6).

5 Days after Injury (Rats 2 and 16). Remnants of necrotic tissue were still to be seen after 5 days, and macrophages, loaded with blood pigment and cellular débris, were more abundant. There were also a few polymorphonuclear leukocytes and lymphocytes. Interstitial fibroblastic and angioblastic proliferation had increased. Dead muscle fibers at the margin of the lesion showed heavy calcification, forming a band of calcium salts (Fig. 3). Beneath this band, the surviving muscle fibers showed granularity of their sarcoplasm with loss of striations, and thin processes had progressed from the ends of these fibers into the network of connective tissue. Columns of two or three nuclei and several mitotic figures were present in these fibers. In deeper portions, many mitotic figures were seen in myocardial fibers (Fig. 10).

6 Days after Injury (Rats 44 and 51). In rat 44, fibrous adhesions to the costal wall had formed by 6 days after injury. In both hearts, the myocardial defect was almost completely filled by young connective tissue which contained many small capillaries. The necrotic tissue had been largely removed, and there were abundant macrophages loaded with cellular detritus, as well as polymorphonuclear leukocytes, lymphocytes, and a few eosinophils. The surviving heart muscle fibers at the border of the lesion showed evident signs of proliferation: the ends were progressing into the connective tissue interdigitating with it; the cytoplasm showed varying staining intensity; there were thin, deeply eosinophilic fibers with columns of two or three nuclei; many fibers and their nuclei showed longitudinal splitting (Fig. 14); several muscle fibers had fused to form multinucleated cytoplasmic masses; mitotic figures were seen frequently in other muscle fibers. In rat 51 no adhesions to the costal wall had formed. The remains of necrotic tissue were more abundant than in rat 44 and, while regenerative processes were present, they were less marked.

7 Days after Injury (Rats 17, 18, and 52). In rats 17 and 18 heavy adhesions to the costal wall had formed in 7 days (Fig. 2). The necrotic tissues had been removed completely in all 3 animals and there were abundant blood pigment-laden phagocytes and mononuclear infiltration. The defect was now filled completely with connective tissue containing abundant capillaries. Rat 17 showed several giant cells of foreign body type in the adhesions. These contained small fragments of black substance, probably carbon from the hot wire. The most interesting findings were at the border of the defect, where the ends of surviving muscle fibers were continuing their progress into the connective tissue. These fibers showed signs of regeneration by both mitotic and amitotic division. Mitotic figures in all phases were seen within myocardial fibers (Fig. 7). The fibers in which the nuclei were undergoing mitotic division had lost their striations, and their cytoplasm had become finely granular. Such fibers were easily distinguished from connective tissue elements by their thickness, and by the color of their cytoplasmic granules when stained by Masson's trichrome method. Amitotic division was represented by longitudinal splitting of the cytoplasm and nucleus in many fibers. In deeper zones, also, the myocardial fibers showed abundant mitotic figures.

8 Days after Injury (Rats 19 and 20). On the eighth day the findings were very similar to those seen on the seventh day, except that the surviving muscle fibers had pushed further into the connective tissue.

9 Days after Injury (Rats 31 and 37). In rat 31, after 9 days, the heart was heavily attached to the costal wall at the lesion by fibrous adhesions. In rat 37 the heart was not attached. Regenerative processes were much more advanced in the animal in which adhesions had formed than in the other. This was believed to be due to the increased blood supply provided the region of the lesion by the

capillaries contained in the adhesions. Nevertheless, in both hearts regenerative activity of myocardial fibers was evident. The ends of surviving muscle fibers at the border of the lesion were progressing into the previously formed network of connective tissue, and longitudinal splitting of their cytoplasm in some areas had given them a frayed appearance. In these fibers the nuclei showed variation in staining intensity, size, and shape, and columns of two or three nuclei were frequently seen in one fiber. Thin, deeply eosinophilic fibers with rod-shaped nuclei were conspicuous. In fibers situated even several millimeters from the lesion and in papillary muscles adjacent to the damaged area (Fig. 16), mitotic division figures in all stages were very abundant. A few macrophages and mononuclear leukocytes were present in the area of the lesion. The interstitial connective tissue gave evidence of proliferation by mitotic figures, mainly around blood vessels.

10 Days after Injury (Rats 33 and 34). No adhesions had formed on either of the hearts examined after 10 days. The defect was completely filled with connective tissue infiltrated by mononuclear leukocytes and a few macrophages. At the edges of the lesion, the muscle fibers showed calcification of various degrees; in some cases this was slight, with only a little granular, basophilic material in the cytoplasm, whereas in others there were large deposits of lime salt. In preparations stained by von Kossa's method, the earlier small deposits were found to be positive (calcium phosphate), but the older and larger deposits were negative (probably another calcium salt). The living ends of the muscle fibers were arrested in some areas by these calcium masses; in fact, very little progress of the fibers into the scar tissue was seen. Nevertheless, mitotic and amitotic division was observed in many fibers.

11 Days after Injury (Rats 21, 22, and 53). In none of the rats examined after 11 days was the heart attached to the costal wall. The defect was filled with well vascularized young connective tissue. No necrotic tissue was seen, and calcification had not occurred in these animals. Blood pigment-laden phagocytes and mononuclear leukocytes were still present in the area of the lesion. The surviving myocardial fibers at the border were progressing into the connective tissue, and amitotic and mitotic division was taking place in many of these fibers. In deeper myocardial fibers also, regenerative activity was obvious from the mitotic figures in different phases.

12 Days after Injury (Rats 5, 27, and 28). The hearts of rats 5 and 28 were attached to the costal wall when seen after 12 days. In all three the defect was filled with young, well vascularized, connective tissue. Lymphocytes and plasma cells were present at the area of the lesion, as well as a few blood pigment-laden phagocytes. The ends of the surviving muscle fibers at the border of the defect were sending thin processes into the connective tissue, and in many of these fibers it was possible to see longitudinal splitting and nuclear changes in the form of rod and horseshoe shaped nuclei. In deeper myocardial fibers abundant mitotic figures in all phases were seen.

13 Days after Injury (Rats 39 and 40). In both rats 39 and 40 the heart was firmly attached to the costal wall at the level of the lesion at 13 days. Well vascularized, young, connective tissue filled the defect. Lymphocytic infiltration and macrophages were still present in the area of the lesion. Thin collagen fibers now were beginning to appear in the connective tissue. The heart muscle fibers at the defect showed evident advancement into the connective tissue, and in some areas these new formed muscle fibers were seen progressing into the adhesions several millimeters beyond the actual border of the heart (Fig. 12). In other areas masses of cytoplasm with several nuclei ("muscle giant cells") were seen (Fig. 15). Longitudinal splitting of myocardial fibers was present at the edges of

the defect. Many nuclear changes, such as columns of two or three nuclei, horseshoe and rod-shaped forms, vesicular nuclei, and mitotic figures were present in myocardial fibers near the lesion (Fig. 9).

14 Days after Injury (Rats 29, 41, and 55). In all 3 animals examined after 14 days, the heart was attached to the costal wall. The defect was filled with connective tissue which was maturing, the fibroblasts were becoming fibrocytes, and collagen fibers were more abundant. Newly formed blood vessels were numerous and endothelial cells in columns were passing from the costal wall through the scar into the myocardium. Lymphocytic and plasma cell infiltrations, as well as macrophages, were still present. Heart muscle fibers penetrating the scar tissue were seen, and amitotic division was evident in some of these fibers, but mitotic activity was greatly decreased, as compared to that in hearts seen somewhat earlier.

15 Days after Injury (Rats 7, 30, and 56). After 15 days, the connective tissue filling the defect was of a more adult type, with collagen and elastic fibers producing retraction of the lesion. Some lymphocytic and plasma cell infiltrations were present. No active regeneration of heart muscle was seen, but in the connective tissue there were many newly formed heart muscle fibers.

20 Days after Injury (Rats 46 and 57). In the 2 rats examined at 20 days, the hearts were not attached to the costal wall. The scar consisted of adult connective tissue containing abundant small blood vessels and limesalt deposits. There was still some mononuclear infiltration. Some of the muscle fibers at the border of the lesion also showed calcification. The scar was somewhat retracted. No regenerative activity of muscle fibers was seen.

25 Days after Injury (Rats 4, 6, and 43). In none of the animals examined after 25 days was the heart attached to the costal wall. The scar was made up of connective tissue with some mononuclear infiltration; few bundles of myocardial fibers were seen in the scar tissue. Small deposits of lime salt were present in the area of the lesion in rat 43.

30 Days after Injury (Rats 8 and 47). The scar was more fully retracted after 30 days and the connective tissue showed more collagen and elastic fibers. Some mononuclear infiltration was still present. There were several small limesalt deposits in the scar.

35 Days after Injury (Rats 9 and 45). An adult connective tissue scar, with abundant blood vessels and bundles of heart muscle fibers within the scar, was found in the rats examined after 35 days.

40 Days after Injury (Rat 48). At 40 days there was a small, somewhat retracted scar, made up of adult connective tissue with abundant blood vessels. A few lymphocytes and mast cells were infiltrating the scar, and bundles of heart muscle fibers were embedded in it.

45 Days after Injury (Rat 50). No changes from the 40-day lesion were seen at 45 days.

50 Days after Injury (Rats 10 and 11). At 50 days there was a very small, somewhat retracted scar, made up of adult connective tissue in which there were small limesalt deposits. Around these, a few giant cells of the foreign body type were seen. Only a few lymphocytes were present.

55 Days after Injury (Rat 54). A mature connective tissue scar with blood vessels was present at 55 days. There were pericardial adhesions with lymphocytic infiltration. Myocardial fibers bridged the defect.

60 Days after Injury (Rats 12 and 13). A small scar, formed by adult connective tissue in which there were bundles of myocardial fibers, marked the site of the lesion in each of the hearts examined after 60 days.

65 Days after Injury (Rat 58). Heavy adhesions to the costal wall had formed

at the level of the lesion in the rat examined after 65 days. In these adhesions there was a large bundle of myocardial fibers which protruded several millimeters beyond the actual border of the heart (Fig. 13). Numerous blood vessels passed from the costal wall through the scar to the myocardium.

70 Days after Injury (Rat 32). A mature connective tissue scar with a few myocardial fibers embedded in it was found in rat 32 which was examined after 70 days. In this animal no adhesions had formed.

75 Days after Injury (Rats 14 and 15). After 75 days there remained only a very small scar made up of adult connective tissue in which there were small deposits of lime salts and a few lymphocytes and mast cells. The pattern of myocardial fibers beneath the lesion was somewhat disorderly.

Controls. Eight newborn rats, 4 to 7 days old, were used as controls, especially in searching for mitotic figures in myocardial fibers. No evidence of nuclear division was found in any of these uninjured hearts.

Summary of Histopathologic Changes in Experimental Animals

Degenerative Changes. Twelve hours after injury the retrogressive changes of the alterative phase of inflammation were apparent. Coagulation necrosis was present in the area of the burn, and in adjacent regions in which the injury was less severe there were granular and vacuolar degenerations of myocardial fibers. In deeper and less damaged fibers, variable degrees of cloudy swelling had developed. Besides these, there were the expected circulatory changes of vasodilatation, congestion, margination of leukocytes, hemorrhages by diapedesis, migration of polymorphonuclear leukocytes through the vessel wall, and extravasation of fluids giving rise to interstitial edema, marking the beginning of the exudative phase of inflammation.

Reparative and Regenerative Processes. After 24 hours the alterative-exudative phase was still more conspicuous, as shown by the presence of necrotic and degenerating tissues, more marked interstitial edema, and infiltration of polymorphonuclear leukocytes, lymphocytes, and macrophages. The early reparative phase of inflammation was represented by beginning removal of necrotic tissues, mitotic activity of the nuclei of interstitial connective tissue at the borders of the lesion, and a few mitotic figures in the nuclei of deeper and less damaged myocardial fibers.

At 48 hours the reparative processes were more conspicuous: the removal of necrotic tissues was progressing rapidly, the proliferation of the connective tissue was more marked, and chains of fibroblasts, many of them showing mitotic figures, were present. At the same time, angioblastic proliferation was evident at the border of the lesion. The mitotic activity in the deeper myocardial fibers was more abundant.

Three and 4 days after the operation, the defect in the heart muscle, which was being created by removal of the débris of damaged tissues,

was beginning to be filled in by fibroblastic and angioblastic proliferation. The macrophages were greatly increased in number and contained engulfed granules of blood pigment and cellular débris. The polymorphonuclear leukocytes were more abundant, and lymphocytes and plasma cells were present. Deposits of calcium salts in damaged heart muscle fibers had already formed. The deeper, less damaged myocardial fibers continued to show regenerative activity in the form of mitotic figures.

Five and 6 days after the operation the necrotic tissues were almost completely removed. This was true particularly in those animals in which adhesions to the costal wall had formed, due probably to a better blood supply to the damaged area provided by new-formed blood vessels in the adhesions. Connective tissue and angioblastic tissue was filling the defect, forming a network in which pigment-laden phagocytes and mononuclear cells were present. The surviving heart muscle fibers at the edges of the defect showed evident signs of regeneration such as the formation of thin processes at their ends which were beginning to progress into the network of connective tissue, the presence of thin, deeply eosinophilic muscle fibers with columns of two or three nuclei, the fusion of muscle fibers forming cytoplasmic masses with several nuclei, the longitudinal splitting of cytoplasm and nuclei of many myocardial fibers, and abundant mitotic figures in all phases of development. All the findings described by Heller[1] and Warthin[2] in diphtheritic myocarditis, as well as abundant mitotic figures in myocardial fibers in the neighborhood of the lesion, were seen in these experimental heart lesions. Mitotic figures in myocardial fibers were seen in the deeper, less damaged fibers at 24 hours after the injury.

During the period from 6 to 14 days after the operation, the necrotic tissue was completely removed. The connective tissue now filled the defect and progressively became adult, with collagen fibers beginning to appear at the 13th day. The ends of the surviving myocardial fibers continued to progress into the new-formed connective tissue and, in some cases in which adhesions to the costal wall were formed, newly formed myocardial fibers were seen, progressing into the adhesions several millimeters beyond the actual border of the heart (Fig. 12). Regenerative activity was seen in all injured hearts during this period, but by the 13th day it was greatly decreased.

Adult Scar. Between 15 and 20 days after the injury, the connective tissue of the scar became of almost adult type, with more abundant collagen and elastic fibers giving some retraction. A few blood pig-

ment-laden phagocytes and some other mononuclear cells were present. Bundles of previously formed myocardial fibers were seen progressing into the scar. Regenerative activity of myocardial fibers apparently had ceased. Limesalt deposits in the scar tissue were seen in several cases. Hearts with parietal adhesions showed abundant blood vessels passing from the costal wall through the adhesions to the myocardium.

From 20 days until 75 days after the injury the scar showed few further changes. The connective tissue filling the defect included abundant collagen and elastic fibers. A few lymphocytes and plasma cells were infiltrating the area even 75 days after the injury, and in some cases a few mast cells were present. Limesalt deposits occurred frequently in the scar, and a few giant cells of foreign body type were seen around them. In almost all of these hearts, bundles of myocardial fibers, which had formed during the period of regeneration, were found bridging the scar. Other muscle fibers were embedded in the scar tissue but did not pass through it and some protruded several millimeters into the adhesions beyond the original epicardial level (Fig. 13).

Discussion

Lesions produced by the application of a piece of heated metal a few millimeters in diameter have been used previously for the study of regeneration of voluntary muscle[37] and cardiac muscle.[35] The advantages are: a fairly uniform lesion can be produced; the difficulties occasioned by the beating of the heart tend to be overcome; and a gradient in respect to degree of damage results, since the deeper fibers suffer less injury, which makes it possible to study several phases of repair and regeneration in each specimen.

The present investigation established that the healing of experimental lesions of this type in young rats proceeds more rapidly than the healing of experimental myocardial infarction in dogs as described by Karsner and Dwyer,[6] and much faster than the healing of human myocardial infarction.[25] This accelerated healing is probably due to a greater power of reparative and regenerative proliferation in young animals and, particularly, to the fact that the myocardial circulation suffers no significant damage. The speed of healing is increased when adhesions to the costal wall are formed, due to the better blood supply thus provided.

The removal of necrotic tissues begins as early as 12 hours after the injury and is completed by the 6th day. Polymorphonuclear leukocytic infiltration is seen in the area of the lesion after 12 hours. It increases in intensity in the next few days and disappears about the

6th or 7th day after the injury. Lymphocytic infiltration begins 48 hours after the injury and is present even 75 days after the operation. Macrophages are observed in the area of the lesion 24 hours after the injury; they contain small fragments of necrotic tissues and blood pigment, and are a constant finding until the 13th day.

Proliferation of the interstitial connective tissue is found 24 hours after the injury at the edges of the lesion and is made evident by fibroblasts showing mitotic figures. They increase rapidly to form a network which progressively fills the defect. On the 13th day, collagen fibers begin to appear and between the 15th and 20th days the connective tissue is sufficiently mature to produce some retraction. Angioblastic proliferation is evident 48 hours after the lesion; it follows the proliferating connective tissue which becomes richly vascularized. When adhesions to the costal wall are formed, small blood vessels pass through them from the costal wall to the myocardium.

Limesalt deposits are formed very often in damaged myocardial fibers at the edges of the lesion, and also in the connective tissue filling the defect. In one heart they were observed as early as 3 days after the injury. This dystrophic calcification is more common when adhesions to the costal wall are not formed. This suggests that probably it is favored by relative local anoxia, which is doubtless more severe if there are no adhesions. Calcium deposits are seen also in the connective tissue of adult scars. The early calcium deposits in myocardial fibers give a positive von Kossa's reaction, establishing that the material is calcium phosphate; the older calcium deposits in the connective tissue of the scar give a negative reaction. Apparently they are not a phosphatic salt of calcium.

The most important finding is the evidence of myocardial regeneration, as demonstrated by mitotic figures in the nuclei of myocardial fibers, by amitotic division of cytoplasm and nuclei of heart muscle fibers around the lesion, and by the advancement of the ends of these fibers into the newly formed connective tissue and even beyond the actual border of the heart into fibrous adhesions between the heart and the costal wall. Myocardial mitotic figures are first observed 24 hours after the injury in the deeper, less damaged fibers of the lesion. They increase in number during the following days and reach a maximum on the 9th day. During this period they are very abundant, four or five being seen in a single low-power field (Fig. 4). Five days after the operation, mitotic figures are seen in the surviving muscle fibers at the edges of the lesion. Mitotic activity is greatly decreased by the 14th day and ceases after the 15th day. Amitotic division of heart

muscle fibers is seen very constantly between the 6th and 14th days. It is made evident by longitudinal splitting of the sarcoplasm and nuclei, and all stages of this process can be observed. Growth of the ends of the surviving myocardial fibers into the network of newly formed connective tissue is first seen 5 days after the injury. This is in the form of thin connective tissue. In older scars, bundles of these newly formed myocardial fibers are seen embedded in the scar tissue, or bridging the defect, or even protruding into the adhesions. Formation of multinucleated cytoplasmic masses ("muscle giant cells") is observed very seldom.

Further experiments are indicated to determine the age to which the regenerative capacity of myocardial fibers is maintained, and also to learn if it is possible to induce regeneration in adult myocardium by injection of extracts of embryonic hearts.

Summary

The literature concerning regeneration of the myocardium is reviewed.

Experimental myocardial lesions were produced in 58 rats, 4 to 7 days old, by the application of a red-hot wire. The animals were killed at intervals from 12 hours to 75 days after injury and the lesions were studied histologically.

Regeneration of myocardial fibers by both mitotic and amitotic division was observed in the period from 24 hours to 14 days after the injury. Newly formed fibers progressed into the connective tissue laid down in the lesion and into the fibrous adhesions to the costal wall which were formed in some of the animals.

I am grateful to Dr. Oscar Duque for the original idea of making this study. I wish to thank Dr. K. Scharenberg for the translation of the Russian article and Dr. J. Bebin for some of the photomicrographs.

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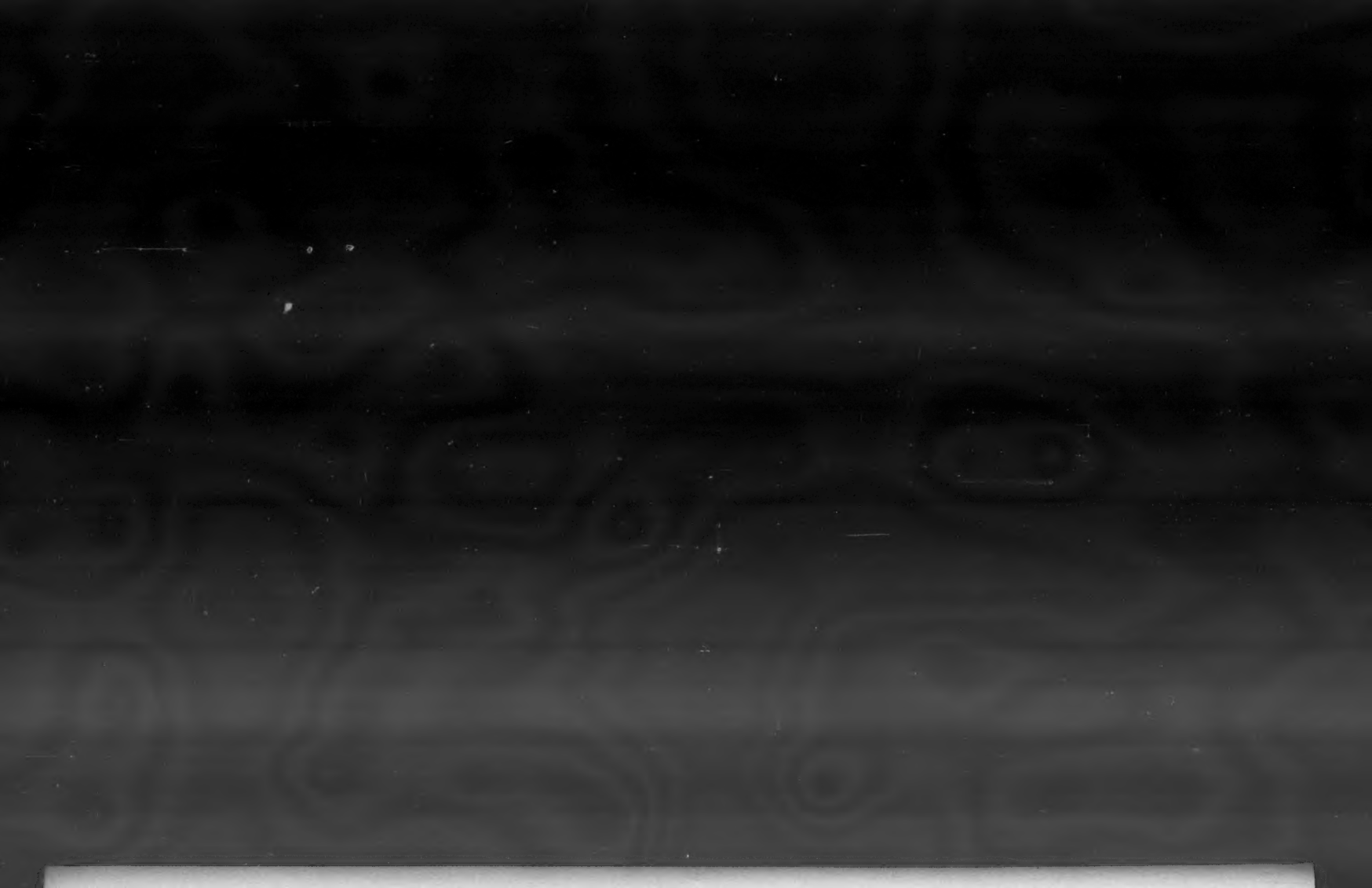
[*Illustrations follow*]

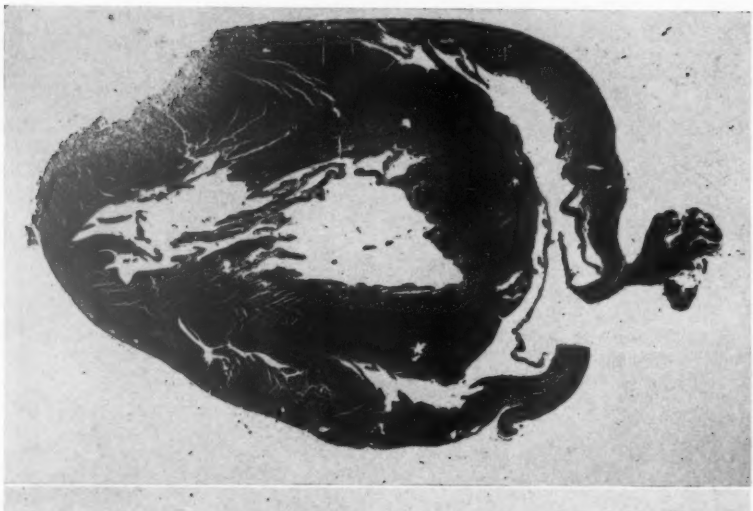
LEGENDS FOR FIGURES

FIG. 1. Heart of rat 35, sacrificed 2 days after the injury. Defect on the anterior surface of the left ventricle near the tip. Hematoxylin and eosin stain. × 15.

FIG. 2. Heart of rat 17, 7 days after the injury. Lesion on the anterior surface of the left ventricle near the tip, firmly attached to the costal wall, which is represented by two pieces of cartilage, voluntary muscle, and connective tissue. Hematoxylin and eosin stain. × 10.

FIG. 3. Rat 2, 5 days after the injury. Calcium deposits in necrotic myocardial fibers at the border of the lesion. Von Kossa's reaction, counterstained with safranine. × 100.





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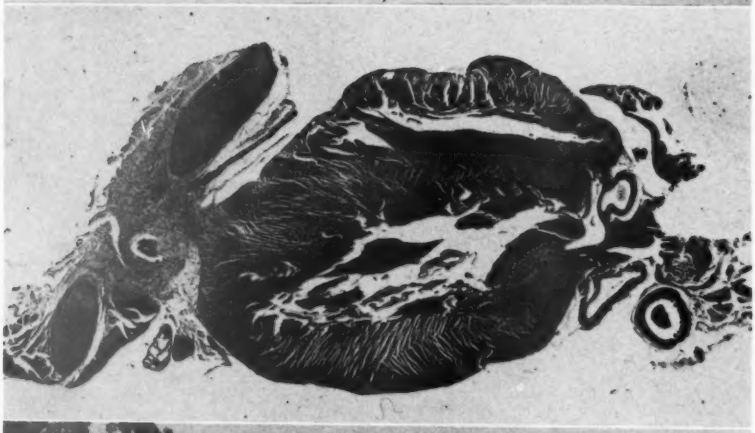

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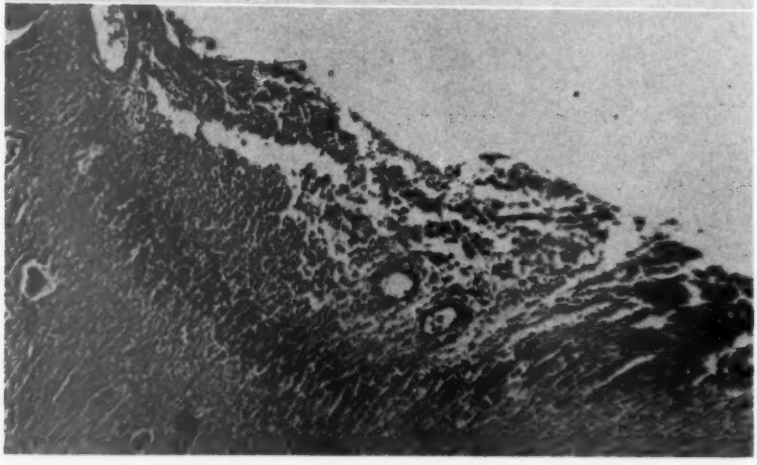

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FIG. 4. Rat 37, 9 days after the injury. Four mitotic figures in myocardial fibers adjacent to the lesion. Masson's trichrome stain. × 420.

FIG. 5. Three of the four mitotic figures of Figure 4 at higher magnification. The chromatin appears as rods and granules. The cytoplasm at the site of nuclear division is clear and granular. Masson's trichrome stain. × 1,350.

FIG. 6. Rat 26, 4 days after the injury. Mitotic prophase in the nucleus of a myocardial fiber, near the lesion. Masson's trichrome stain. × 1,350.

FIG. 7. Rat 7, 7 days after the injury. Metaphase in the nuclei of two myocardial fibers near the lesion. Masson's trichrome stain. × 1,350.

FIG. 8. Rat 36, 2 days after the injury. Mitotic figure in a myocardial fiber located in the deeper portion of the lesion. Masson's trichrome stain. × 1,350.

FIG. 9. Rat 40, 13 days after the injury. Mitotic figures and vesicular nuclei in myocardial fibers near the lesion. Masson's trichrome stain. × 1,350.

FIG. 10. Rat 16, 5 days after the injury. Anaphase in the nucleus of a myocardial fiber. Masson's trichrome stain. × 1,350.

FIG. 11. Rat 37, 9 days after the injury. Myocardial fiber in a zone adjacent to the lesion, showing two nuclei, probably after mitotic division. Masson's trichrome stain. × 1,350.

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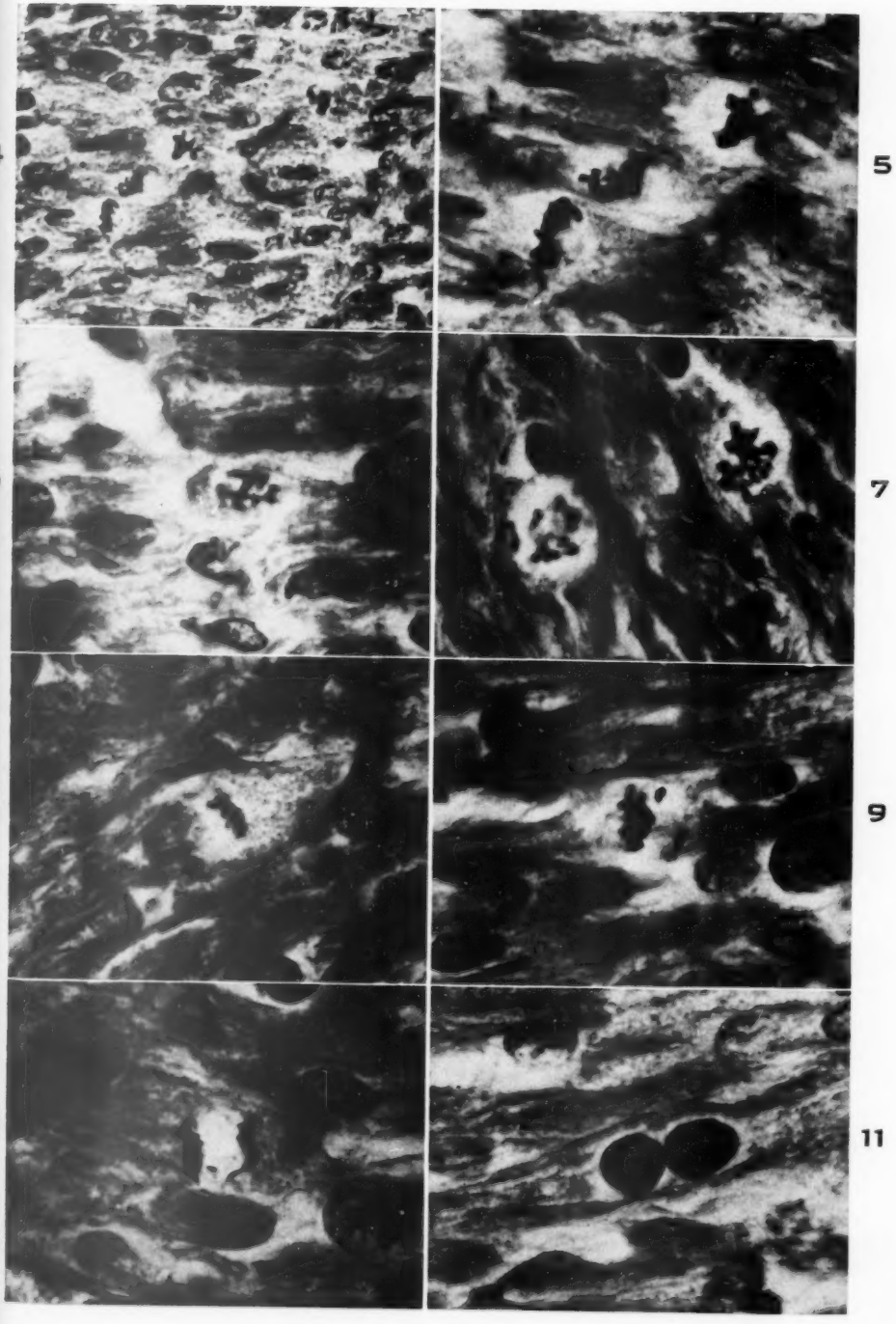

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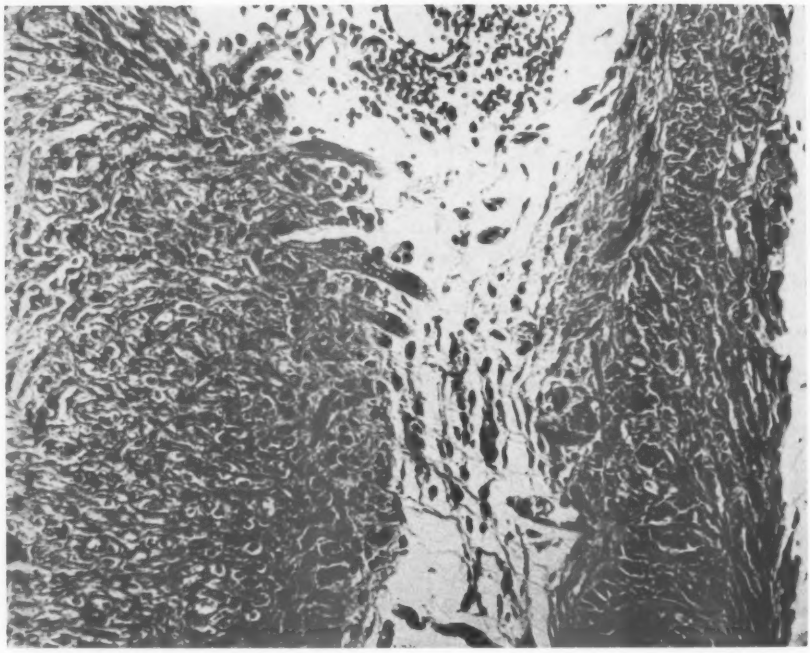
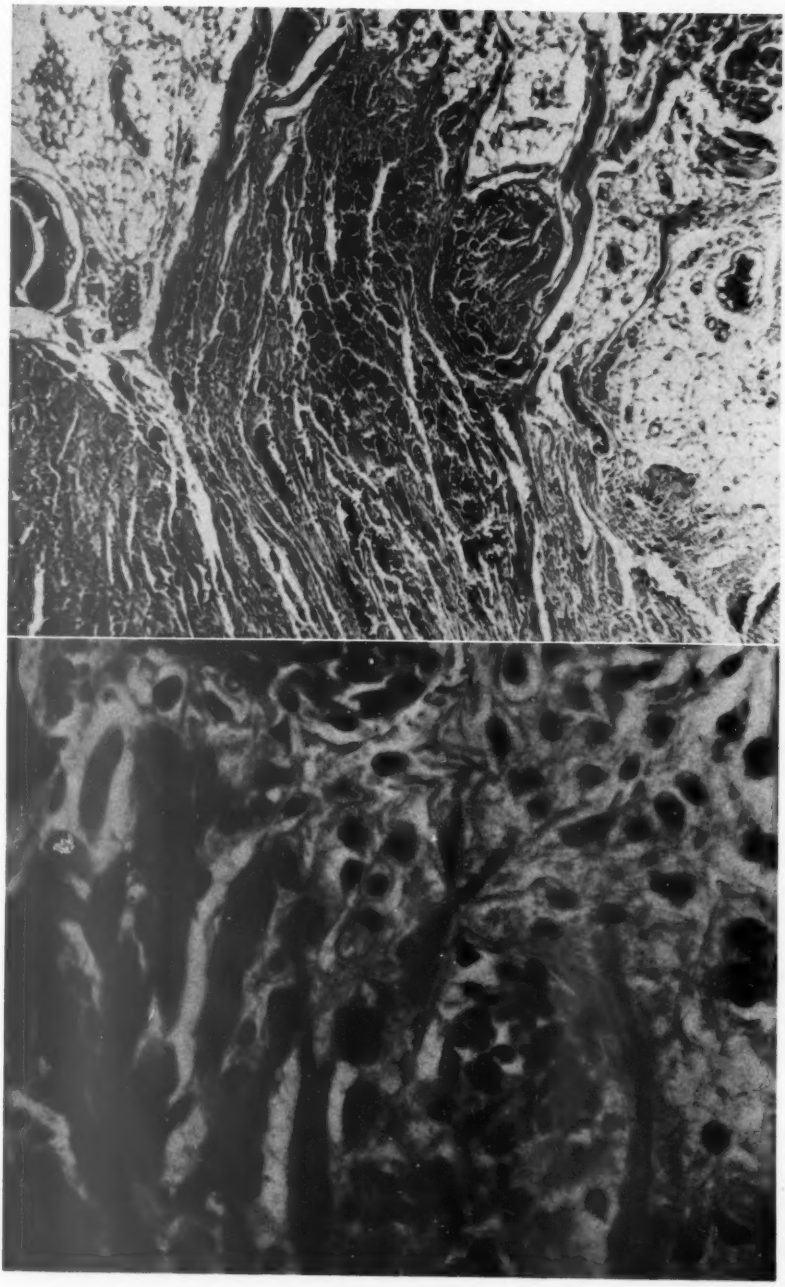


FIG. 12. Rat 40, 13 days after the injury. The heart is at the left, the costal wall at the right; between them are young fibrous connective tissue adhesions. Several myocardial fibers are progressing into the adhesions beyond the actual border of the heart. Masson's trichrome stain. × 330.

FIG. 13. Rat 58, 65 days after the injury. A large bundle of myocardial fibers protrudes beyond the border of the heart into the adhesions. Columns of blood vessels pass from the costal wall (upper right corner) through the adhesions to the myocardium. Masson's trichrome stain. × 330.

FIG. 14. Margin of the lesion of rat 44, 6 days after the injury. The ends of the surviving myocardial fibers show thin processes extending into the young connective tissue filling the defect. There is longitudinal splitting of some myocardial fibers. Masson's trichrome stain. × 700.



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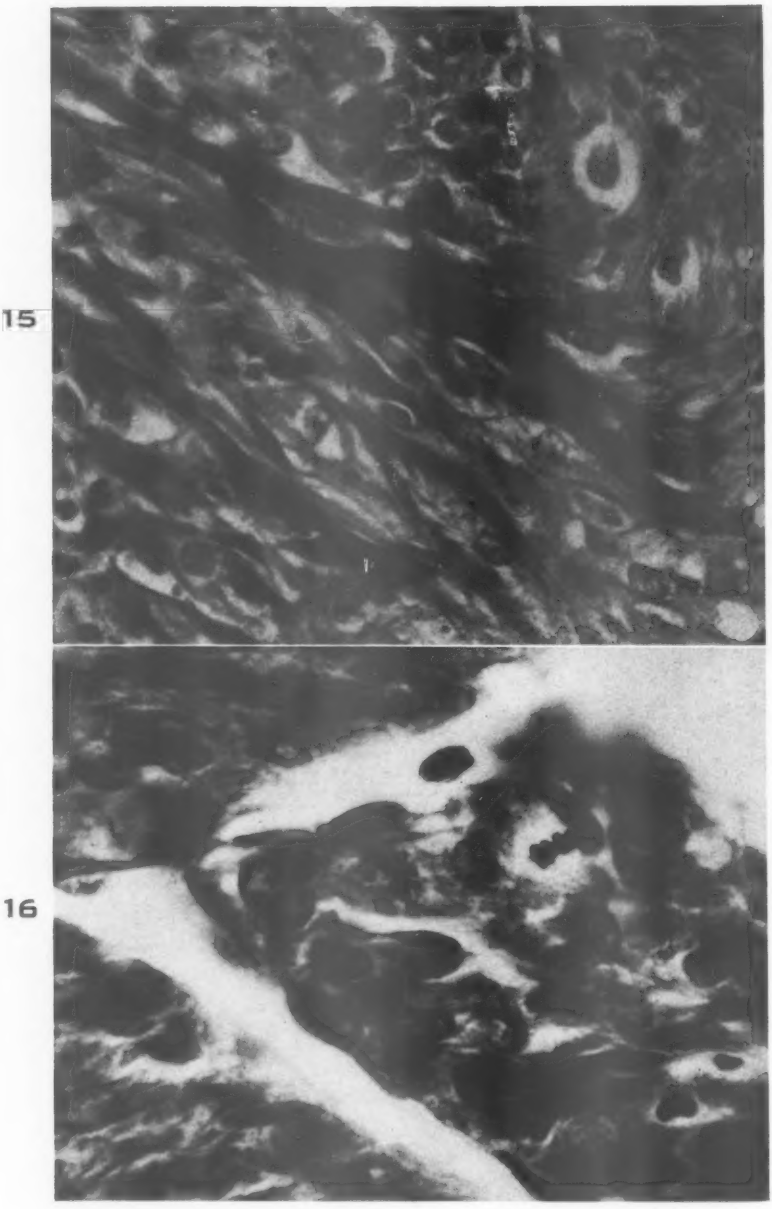

FIG. 15. An area of the margin of the lesion of rat 39. There has been fusion of several myocardial fibers to form a cytoplasmic mass containing several nuclei, one of which shows mitotic division. Masson's trichrome stain. × 700.

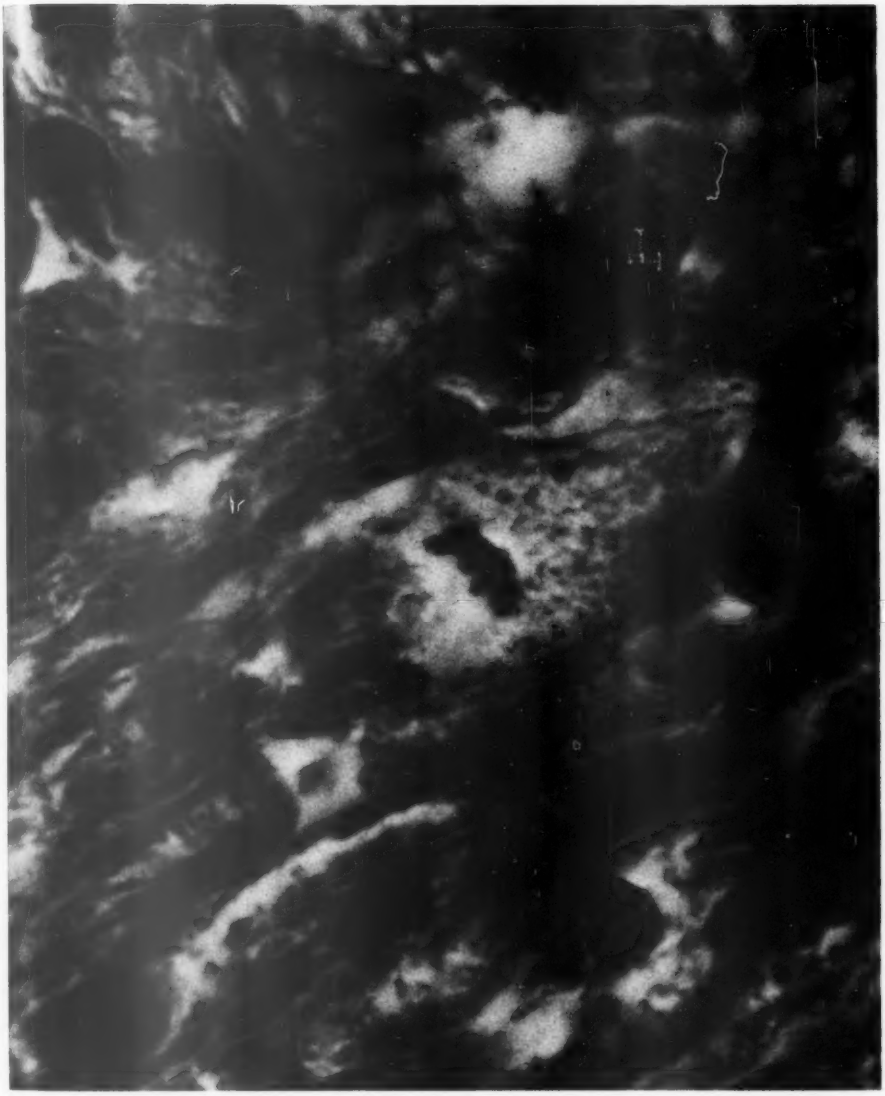

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FIG. 16. Rat 37, 9 days after the injury. Two mitotic figures in a papillary muscle adjacent to the lesion. Masson's trichrome stain. × 1,350.

FIG. 17. Rat 36, 2 days after the injury. Higher magnification of Figure 8. The sarcoplasm at the site of nuclear division is clear and finely granular. Masson's trichrome stain. × 2,700.

THE PATHOLOGY OF CHRONIC ARTHRITIS FOLLOWING NATURAL AND EXPERIMENTAL ERYSIPELOTHRIX INFECTION OF SWINE *

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Arthritis of swine occurs commonly under farm conditions. Bacteria are found frequently in affected joints; however, no infectious agent can be isolated from others. In young pigs pyogenic bacteria frequently are found. It is believed that pyogenic infection occurs at birth, presumably through the umbilicus.

In joints infected with *Erysipelothrix rhusiopathiae,* pus usually is absent but extensive proliferative changes may be present. Loeffler[1] is credited with first observing *Ery. rhusiopathiae* in swine. He found it in the blood of a pig which apparently died from erysipelas.

Natural *Ery. rhusiopathiae* infections have been reported in sheep, cattle, horses, turkeys, ducks, peacocks, pheasants, and farm-raised mink (Beaudette and Hudson,[2] Graham *et al.*,[3] Marsh,[4] Paterson and Heatley[5]). In some of these animals, particularly cattle and sheep, the infection is associated with arthritis. Human infections have resulted from handling fish, infected animals, and bones according to Klauder and Harkins,[6] Morrill,[7] and McGinnes and Spindle.[8]

Collins and Goldie[9] reported on naturally occurring arthritis in swine. Two healthy pigs put in contact with the arthritic animals developed arthritis. Bacteriologic examinations failed to determine the etiology in the naturally occurring cases, but agglutinins for *Ery. rhusiopathiae* were found. Two pigs were each given 2 cc. of a 24-hour broth culture of *Ery. rhusiopathiae* subcutaneously. Two other pigs received similar doses and 2 weeks later a repeat dose. The pigs were killed 1 week after injection and showed no evidence of arthritis. Later 4 pigs were each given repeated intravenous injections of 2, 5, and 2.5 cc. of a 24-hour broth culture at 2-week intervals. Four other pigs

* This investigation was supported in part by research grant A-45(C2) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

Submitted as Journal Paper No. 915, Agricultural Experiment Station, Purdue University.

Received for publication, February 9, 1956.

† Author of thesis: The Pathological Effects of the Bacterium *Erysipelothrix rhusiopathiae* on Swine, submitted to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August, 1954. Now at Department of Pathology and Parasitology, School of Veterinary Medicine, University of Georgia, Athens, Ga.

each received 5 cc. of the broth culture subcutaneously, then were given 5, 5, and 7 cc. at 2-week intervals. Pronounced polyarthritis developed in all 8 animals.

The object of the work reported here was to study the pathologic effects of *Ery. rhusiopathiae* in swine, particularly in regard to arthritis. Naturally occurring arthritis in swine has pathologic features similar to those observed in man. It may be that information on arthritis in swine will aid in a better understanding of the condition in man.

Materials and Methods

Sixteen swine were used, 9 of which were naturally occurring field cases of erysipelas and 7 were experimental animals. The naturally occurring cases came from three different droves. Three cases came from a drove in which an acute outbreak was occurring. Three chronic cases came from each of the other two droves.

The experimental swine were from a pure-bred Chester White herd in which erysipelas was not known to have occurred since the herd was established in 1941. All animals had been vaccinated for hog cholera with anti-hog cholera serum and virus when 8 weeks old. All were between 3 and 4 months of age, and were in excellent health when the experiment was started.

Soft tissues for histopathologic examination were taken at necropsy and fixed in 10 per cent formalin. Paraffin sections were stained with hematoxylin and eosin. Bone specimens were decalcified according to Lillie's method,[10] sectioned, and stained with hematoxylin and eosin.

Results

Erysipelas and Arthritis Following Contact with Acute Naturally Occurring Cases

Three acute cases of swine erysipelas were obtained. One animal was slaughtered and bacteriologic cultures made to confirm the diagnosis. Two normal shoats were placed in contact with the remaining animals. Body temperatures of the contact shoats were elevated at 72 hours following exposure and reached 107.5° and 108° F. at 96 hours, gradually returning to normal (100.4° to 104° F.) by the eighth day. Both contact animals developed chronic polyarthritis which persisted for 1 year, when they were killed. At necropsy there was marked enlargement of the carpal and tarsal joints and obvious proliferative coxitis. The kidneys were fibrotic and the adrenal glands were enlarged and contained some cysts. All other viscera appeared normal. There was extensive pannus formation in the carpal and tarsal joints. However, bacteriologic examination of these joints gave negative results.

One of the swine with naturally occurring erysipelas died 2 days after arrival at the laboratory. *Ery. rhusiopathiae* was isolated from the kidneys, liver, and lymph nodes. This culture was used in other experiments to be reported. The other swine with naturally occurring erysipelas developed chronic arthritis which persisted for 1 year, when they were slaughtered. The pathologic changes were similar to those found in the contact animals.

At necropsy 1 year later, the 6 other swine with naturally occurring chronic polyarthritis had gross lesions similar to those described for the contact animals. Bacteriologic examination of these joints gave negative results.

Intravenous Inoculation with a Smooth Colony of Ery. rhusiopathiae Isolated from the Acute Septicemic Field Case

Each of 5 shoats received 3 cc. of a 24-hour culture in the anterior vena cava. Two of the animals developed temperatures of 107° to 108° F. by the fourth day and died of erysipelas on the fifth day. *Ery. rhusiopathiae* was isolated from lymph nodes, pancreas, brain, heart's blood, liver, kidneys, spleen, and adrenal glands.

The third animal developed chronic arthritis and necropsy was performed 10 months after exposure. At 100 days the animal had enlarged carpal and tarsal joints and was very lame. Radiograms at 7 and 9 months showed narrowed joint spaces, early osteophyte formation, and apparently some rarefaction of the bones (Figs. 1 and 2). The distal ends of the radius and ulna seemed slightly enlarged. At necropsy the kidneys were enlarged and pale. The adrenal glands were markedly atrophic. All other viscera appeared normal. The carpal and tarsal joints were much enlarged and firm. Most of the joint enlargement was due to fibrosis. The joint capsules were thickened. Radiograms showed that some bony proliferation had occurred. Pannus formation and some erosion of the articular cartilage were present. Bacteriologic examination of the carpal and tarsal joints gave negative results.

The 2 other animals showed a mild transient febrile response and recovered.

Observations on Pathologic Changes

In acute arthritis there is synovitis characterized by vascular engorgement and edema of the synovial tissues. The effusion is turbid or serosanguineous and mucinous. The synovial villi show evidence of beginning proliferation, and beginning lymphocytic infiltration (Fig. 3). *Ery. rhusiopathiae* was isolated from joints in this stage.

As the arthritic condition progresses, greater proliferation and less edema are observed. Proliferation of the mesothelial cells covering the hypertrophied synovial villi is present. The villi contain young, highly

vascular connective tissue, plasma cells, and lymphocytes (Fig. 4). Collins and Goldie[9] found the greatest cellular collections just beneath the synovial lining. In our material the greatest collections occurred in the hyperplastic synovial villi. A striking feature was the accumulation of dense collections of lymphoid cells.

Synovial villi in carpal and tarsal joints frequently resembled granulomatous polyps (Fig. 5). Very few polymorphonuclear leukocytes were observed. The infiltrating cells were principally lymphocytes, with some plasma cells and eosinophils. Little, if any, suppuration was observed. The destruction of cartilage and bone of suppurative arthritis was not found. The synovial effusions were non-purulent, and contained mostly monocytes, lymphocytes, and a few mesothelial cells. Lymphocytes were the most numerous and in fluid from eight of twelve joints examined they were the only cells found.

In advanced chronic arthritis with pannus formation of subchondral origin, fibrosis in the adjacent marrow spaces, increased vascularity, and collections of lymphocytes occurred. In cases in which the pannus apparently originated from the capsule, no subchondral fibrosis was found (Fig. 6). Pannus attachment to the articular cartilage was accompanied by erosion of the cartilage. In advanced cases, in which little or no synovial fluid was present, intra-articular fibrous adhesions had formed (Fig. 7), with fibrotic thickening of the joint capsule. No microscopic differences were observed between the naturally occurring cases and the experimentally produced cases of chronic polyarthritis.

Discussion

Sequence of Events in Ery. rhusiopathiae *Infections*

Bacteria, entering the body, are presumably picked up by the lymphatics and carried to the nearest regional lymph node where infection is established. The organisms then spread to other lymph nodes and finally enter the blood stream, resulting in bacteremia. Soon the infection is established in the joints. Edema and congestion of the synovial membranes occur, followed by proliferative changes in the intra-articular structures. Suppuration rarely occurs, and when it does it is focal. Numerous lymphocytes and plasma cells invade the area with a limited number of polymorphonuclear leukocytes and a few eosinophils. Eventually the synovial membrane becomes converted into a form of granulation tissue, and individual villi resemble granulomatous polyps. The synovial lining cells rarely are lost. The synovial fringes probably combine with proliferating perichondrium to form a pannus. The articular cartilage becomes eroded superficially in the areas of pannus attachment and deeply as a result of the in-

flammatory reaction in the subchondral bone. Bacteria may no longer be demonstrable in the joints but the arthritic process may continue unabated.

Collins and Goldie[9] believed that ultimately one of three things may happen in advanced chronic arthritis of swine in which no infectious agent can be isolated. (1) The lesion may resolve and the joint recover. (2) Healing of the damaged tissues may take place by fibrosis resulting in formation of intra-articular adhesions. (3) The synovial granulation tissue may assume some of the properties of a benign neoplasm and proliferate in the absence of the exciting microorganism. Thus, the arthritis is maintained in a state of clinical activity. The results of our experiments support this last belief, especially in the case of the continued clinically active form after the joints apparently are sterile. It may be that renal and adrenal damage play a part in these cases of advanced chronic polyarthritis.

Macroscopically and microscopically no pathologic difference was noted between the naturally occurring field cases of chronic polyarthritis and those experimentally produced by Erysipelothrix infection. It is believed that the naturally occurring cases were originally due to Erysipelothrix infection even though bacteriologic examination gave only negative results at necropsy. We[11] were not able to isolate *Ery. rhusiopathiae* from the joints of swine with chronic arthritis 226 days after exposure, even when the animals were severely affected.

A comparison of arthritis of swine and rheumatoid arthritis in man shows some similarities. According to Collins and Goldie,[9] the pathologic changes in the joints show clear macroscopic and microscopic similarities. The changes are essentially proliferative in both diseases, non-suppurative, and have a tendency toward granulomatous proliferation in the synovial membrane. Pannus formation and focal accumulations of lymphocytes are observed in both diseases. Destruction of cartilage at the site of pannus attachment and subchondral cellular reaction appear similar. Rarefaction of the bone was revealed by radiography, and intra-articular fibrous adhesions are common to both diseases. Collins and Goldie[9] believed that no further analogy can be drawn between the two diseases until the cause of rheumatoid arthritis in man is known.

Summary

A naturally occurring arthritis in swine has been studied. Bacteriologic and pathologic studies were made of 6 cases of chronic arthritis and 3 acute cases of swine erysipelas.

A similar arthritis developed in 2 shoats which were placed in contact with 2 swine with naturally occurring acute swine erysipelas.

Two of 5 shoats injected intravenously with 3 cc. of a 24-hour culture of the organisms died of acute swine erysipelas by the fifth day. The third shoat developed advanced chronic polyarthritis, while the other 2 resisted exposure.

In animals experimentally infected with *Erysipelothrix rhusiopathiae*, a chronic proliferative polyarthritis developed, with pannus formation. The productive reaction was of both subchondral and marginal origin. Degenerative changes in the adrenal gland and fibrotic kidneys were found in advanced cases of chronic arthritis.

The pathologic changes in the joints of arthritic swine resemble rheumatoid arthritis in man in many respects.

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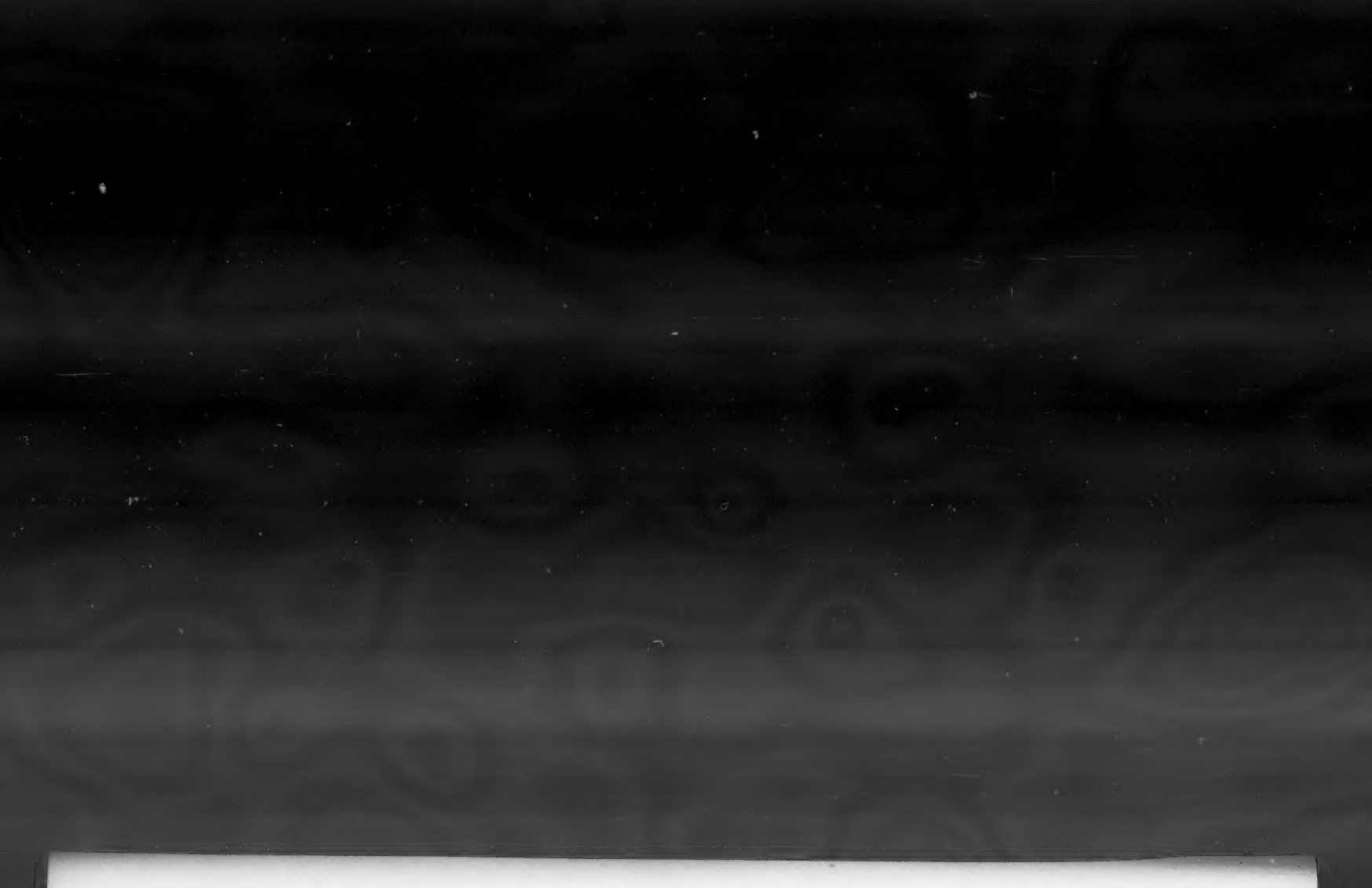
LEGENDS FOR FIGURES

FIG. 1. Roentgenogram of the carpus of a hog 7 months after exposure to *Erysipelothrix rhusiopathiae,* showing narrowing of the joint spaces and osteophyte formation.

FIG. 2. Roentgenogram of the carpus of the same hog from which Figure 1 was taken, 9 months after exposure to *Ery. rhusiopathiae,* showing obliteration of joint spaces, ankylosis, and osteophyte formation.



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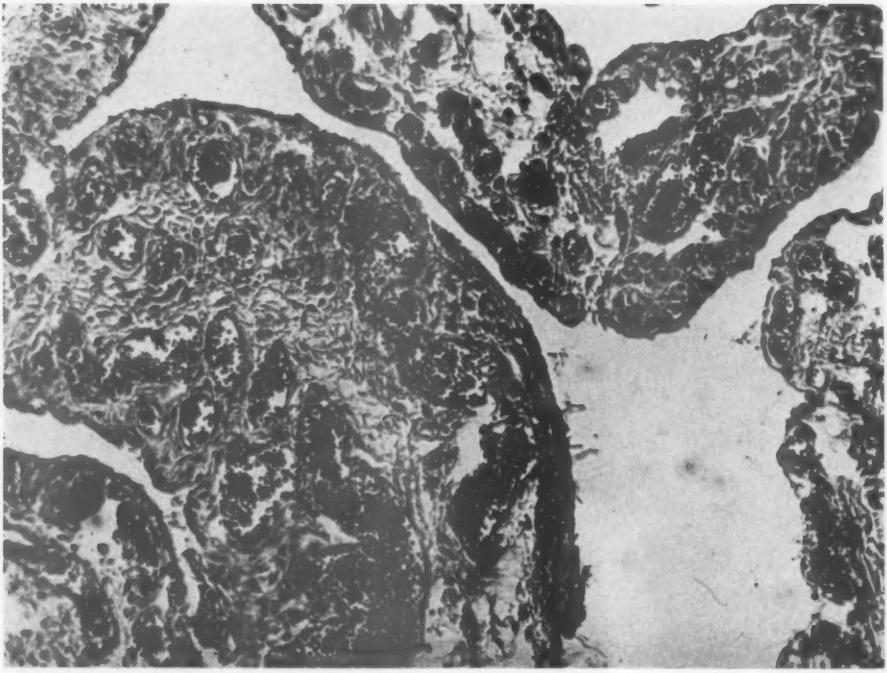

FIG. 3. Marked hyperemia of synovial villi in experimentally produced acute arthritis. × 185.

FIG. 4. Beginning proliferation of the synovial villi with focal round cell accumulations in experimentally produced arthritis. × 185.

FIG. 5. A synovial villus from a case of advanced chronic arthritis, showing some granulomatous changes in addition to lymphoid cell infiltrations. × 185.

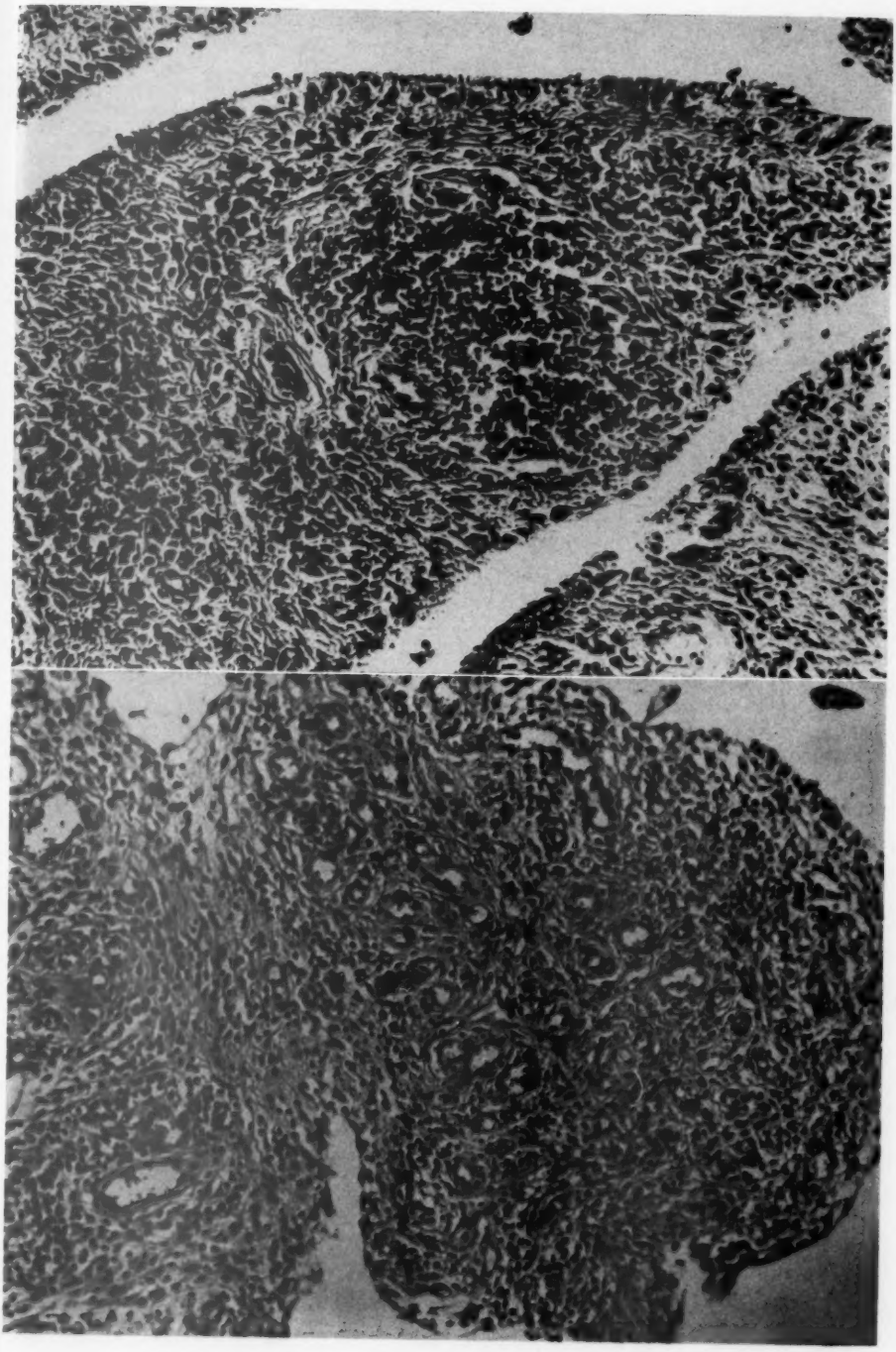

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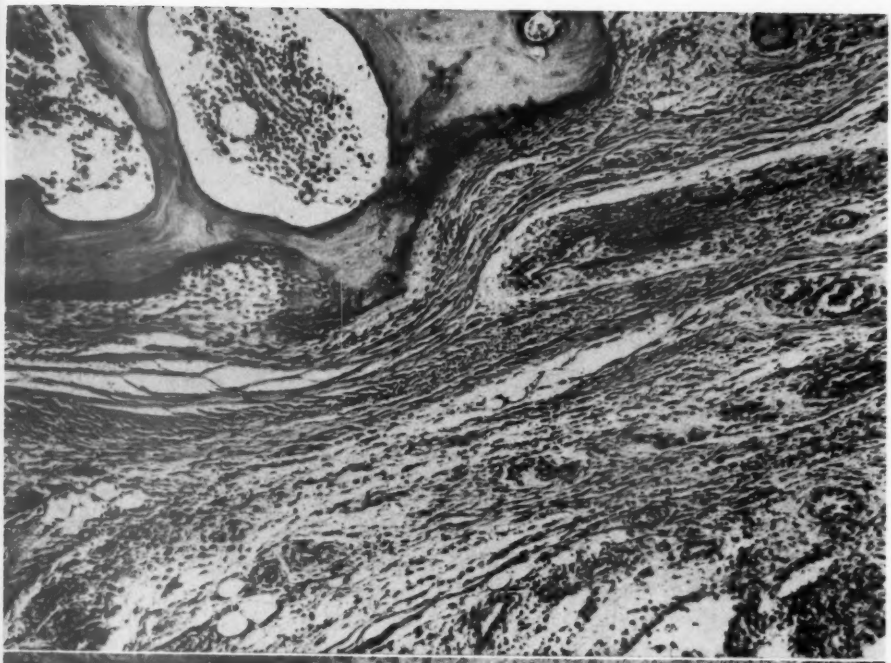
FIG. 6. Extensive pannus formation originating from the synovial membrane. Destruction of the articular cartilage is almost complete. × 85.

FIG. 7. Fibrous ankylosis of a joint in advanced chronic arthritis. × 185.



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THE FUNDUS OCULI AND THE DETERMINATION OF DEATH *

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Pathologists and clinicians alike are made aware all too frequently of the need for a simple and readily available method for determining whether death has occurred. While a condition of "suspended animation" does not exist in the literal sense, respiratory and circulatory activity may be so reduced in both rate and intensity as to escape detection by the usual procedures. Because the possibility of resuscitation may be at stake, time may not permit the use of the electrocardiograph or similar apparatus. In addition to establishing the fact of death, reasonably accurate determination of the moment of death may be of prime significance in the subsequent adjudication of the descent of property.

It has long been known that complete cessation of blood flow to the bulbus oculi results in rapid and striking morphologic changes in the retina and its vessels. These changes were first studied by Bouchut[1] (1863), who advanced them as constituting the earliest and most accurate sign of death. Many subsequent investigators have corroborated this assertion. Several, in addition, have either intimated or stated emphatically that such changes unequivocably indicate cardiac standstill; and thereby have shifted attention somewhat from their mortal significance to the possibility of their serving as a guide to resuscitability.

It is my purpose to emphasize, as others have done previously, the importance, speed, accuracy, and ease of observation of the fundus oculi; to summarize all preceding investigations; to illustrate photographically the appearance of the retina during and after death; and to correlate the illustrated fundic changes with simultaneous electrocardiographic tracings.

Changes in the Fundus with Cessation of Cardiac Activity

Three cardinal changes, occurring more or less concomitantly but at different velocities and with different degrees of intensity, comprise the so-called ophthalmoscopic signs of death. In reality, these are actually signs of cessation of blood flow to the bulb and, inferentially, of cessation of cardiac output.

* Received for publication, April 25, 1956.

Disappearance of the Central Retinal Artery and Its Branches. As the circulation slows down with gradual failure, the arterial blood columns become "granular"[2,3] due to visibility of clumped, rapidly moving erythrocytes. They may eventually form "beads," or become "striated" at the instant of heart standstill; at the same time the caliber becomes markedly reduced. As a rule the arterial pattern disappears rapidly, perhaps within the following minute or less,[4-6] although it is reported to have persisted for much longer periods with the initial onset of peripheral arteriolar haziness.[7-10] Arterial blood, when seen, remains brighter than that in the veins.[8]

Color Changes in the Retinal and Choriodal Coats. The retina and choroid normally have an intense reddish color due to the rich network of choroidal capillaries which can be seen easily through the transparent retina. At the instant when circulation stops, the choroid becomes pale yellow-orange,[7-11] and the optic disk, normally a pale rose color,[5] immediately becomes ghastly white and more sharply outlined against the now paling retina.[5,8] Grayish retinal turbidity begins to appear rapidly,[8,9] at first in the posterior pole of the globe around the disk, gradually progressing to the periphery where some red tinting may be perceptible even hours after death. The macula appears as a red spot on a field of delicate gray-yellow, then gray-white, and deep gray after several hours; the amount of pigment, which varies with race and individually, determines the degree of yellow or brown overlay.

Retinal Venous Changes. As in the arteries, circulatory deceleration results in "granularity" of the blood columns flowing through retinal veins. Almost without exception these columns break into segments ("beads,"[2] "striations"[3]) within a few seconds after circulatory stagnation.[2,5,6] Such formations are seen chiefly in the larger veins on or near the disk,[8] although the site is variable.[3] The segments and intersegmentary clear spaces continue to move smoothly for approximately 10 to 20 minutes after their evolution[6] and finally become completely static. They remain visible in this fashion for several hours. During this period they usually return to their original shape and position after external pressure upon the globe, which causes only temporary blanching.[3] Hours later the final picture is one of magenta colored venous segments on a uniformly gray retina which shows no trace of an artery. These striking vascular phenomena, especially the rapidly occurring venous segmentation, constitute the less subtle and more objectively cogent components of the ophthalmoscopic triad.

Historical Review

Just 1 year after Helmholtz invented the ophthalmoscope, the first documented studies on mortal retinal changes were conducted by E. Bouchut,[1,12,13] (1863) who, under the pseudonym of Pierre Durand, received a prize offered by the Marquis d'Ourches for the most nearly infallible sign of death. He stressed rapid disappearance of retinal arteries, the uniformly pearl-gray fundus, and the so-called pneumatosis or segmentation of venous columns, which results, in his opinion, from immediate post-mortem release of gases present in the blood. He supported this contention by referring to frequent gaseous segmentation of meningeal vessels observed at necropsy as manifesting a generalized somatic phenomenon. These findings were reported by him to the Academy of Science, Paris, in 1864 and first published in 1868 ("A Memoir on Some New Signs of Death Furnished by the Ophthalmoscope"). Bouchut[1] also published another brief account in 1876 together with the earliest extant illustration, a single colored drawing reproduced here as Figure 8.

Meunier[14] (1868) and Poncet[15] (1870) verified the above observations. The latter, working independently and ignorant of Bouchut's efforts,[1,4] noted absence of the characteristic signs in syncopic patients. By 1873 many observers had become sufficiently skilled in ophthalmoscopy to observe the intra-ocular alterations at death. On the other hand Weber,[16] in 1876, noted persistence of intact arterial blood columns along with venous blood segmentation. Becker,[17] too, noted full arteries. Gayet[7] studied eyes immediately after decapitation and observed orange-yellow fundi (not described by Bouchut), thin arterial remnants, and irregularly occurring peripheral "beading" of venous blood. Also, he reported that in two bodies which had remained for several hours at a low temperature (6° to 10° C.) after death, the retinal vessels appeared normal. He therefore concluded that retinal vascular signs are not pathognomonic of death. Doe[18] insisted that they are.

Usher[2] (1896), working with humans, monkeys, dogs, cats, and rabbits, correlated not only the "granularity" of retinal circulation with a generalized circulatory deceleration, but also the eventual "beading" of venous blood and the disappearance of arteries with complete cardiac standstill. He was the first to state that "granular movement," a result of ophthalmoscopically magnified (20 ×) individual and clumped red cells, does not necessarily mean death, and that recovery is possible. Absence of fundic signs in an excessively

chloroformed, apparently dead monkey, which recovered unexpectedly, served to emphasize his point. He devised experiments to implicate a relative increase in intra-ocular pressure as a cause of "beading"; and he insisted, contrary to Aldridge,[19] that retinal arteries empty retrogradely after death.

In the face of conflicting opinions of contemporaries, Gayet[7] (1900) concluded that further study led to less dogmatism about infallibility of the signs and that ". . . the former condition of the patient's health, certain individual peculiarities, the mode of death, and, finally, the surroundings in which the body is kept . . ." are all factors which influence the significance of any ocular sign. Gowers[20] (1904) briefly mentioned the signs and cited Gayet's work.

Albrand,[8] in 1904, emphasized the purely mortal aspects of retinal changes and presented the detailed descriptions of opacification of the media as well as of retinal color changes after death. He stated that arterial blood, which usually disappears quickly after death (within 5 minutes at most, but usually much earlier), is of lighter color than venous blood which tends to become gradually darker as tissues usurp liberated oxygen. Irregularly narrowed veins persist for hours, with coagulated segments seen early in the physiologic depression, separated by fluid and by fine strands of erythrocytes. He included colored drawings of each eye of a cadaver at 45 minutes and at 6 hours after death, the former showing persistent, narrowed arteries and veins (segmented on the disk) on a yellow-orange fundus; the latter, only two dark venous trunks near the papilla upon a gray background. Albrand asserted that these changes allow for conclusive determination of onset of death, time of death, position of the body at death (congestion producing a lingering red tinge in the more dependent eye), and the type of death involved, either rapid or slow. His criteria for the last point are questionable.

In 1906 Albrand-Sachsenberg[9] placed emphasis on the use of funduscopic signs in determining resuscitability. If even the feeblest heartbeat and a barely vital circulation are present, regardless of all appearances of death, the characteristic funduscopic changes do not occur; arterial volume is usually markedly reduced and the arteries are traversed by pulsation waves coincident with heartbeats. Such may be the case with severe anemia, syncopal conditions, pathologic sleep, and electrocution (which often produces an ominous, immediate, but transient and completely reversible state of apparent death). Albrand-Sachsenberg argued that it is the duty of every physician who examines corpses to differentiate real from apparent death with the

ophthalmoscope, to institute resuscitative measures if indicated, and to obviate by frequent examinations the premature abandonment of resuscitative efforts or the remote possibility of premature interment. Although funduscopic signs may vary in detail from case to case, he was convinced that the over-all picture is sufficiently distinct, even with severe concomitant changes of diabetes, nephritis, or arteriosclerosis, to warrant its universal application. Albrand-Sachsenberg conceded that emboli occluding the central retinal artery during life also may bring about similar retinal changes, but at a slower rate.

Kahn[3] (1913) was concerned with venous segmentation which he attributed to post-mortem coagulation and the squeezing out of serum to accumulate between the coagula. He rejected the "pneumatotic" hypothesis and devised various *in vitro* experiments to substantiate his views. He discussed briefly 3 cases which manifested venous segmentation at varying intervals after death and in various retinal quadrants.

On the basis of 12 cases, Würdemann[10] (1920) considered that the most pronounced and pathognomonic sign is a sickly yellowish discoloration of the fundus and blanching of the disk as heart action diminishes. Arterioles become constricted, straighter, and disappear in approximately ½ hour after death, at which time venous segmentation becomes evident due to coagulation of blood. Thus one has positive evidence of death plus an indication of elapsed time after death. Two colored drawings show most of these changes, but segmentation is not represented.

Ginestous and Lande[4] (1929) observed retinal changes after decapitation, described the classical color changes, and illustrated diagrammatically the venous segmentation which they compared to the appearance of bubbles of air in a colored-alcohol thermometer. They attributed this change to the instantaneous release of gases at death and even suggested the possibility of immediate putrefaction.

Pines[11] (1931) stated that blood "breaks up" in the arteries in 15 to 20 minutes after death, and that this occurs later in the veins. All this, he admitted, was contrary to textbook descriptions and purportedly a result of post-mortem regurgitation of static blood through a dilated aortic valve. He preferred to think of intersegmentary vascular spaces as being filled with air ("pneumatosis"). Royo-Villanova Morales[5] (1934) was in agreement with others as to color changes and maintained that only veins, segmented by bubbles of air, remained after death.

The most recent significant contribution was that of Salsbury and

Melvin[6] (1936). After observations on moribund patients and well planned experiments on dogs, they concluded that slowing of the general circulation produces "granularity" of the retinal venous blood stream; that absolute cessation of blood flow to the globe, as by bilateral carotid arterial occlusion, proximal aortic occlusion, cardiac arrest, or ventricular fibrillation, is necessary for the disappearance of the arteries and segmentation of venous columns within a matter of seconds; that venous segments continue to move toward, and fall strikingly over, the edge of the physiologic depression for at least 10 minutes *after complete stagnation* of blood; and that functional restoration of circulation totally abolishes these alterations in a few seconds. Emphasis was centered upon use of the signs—above all, the moving segments—as a clinical guide in resuscitation. They concluded by citing the need for electrocardiographic correlation with these changes.

Materials and Methods

Ophthalmoscopic photography dates back at least to 1899[21] at which time photographs in black and white were, in general, equal to the best drawings, especially when touched up with water colors.[8] The type of ophthalmoscopic camera used for the present work was evidently devised by Mawas[22] in 1933 and built by Nordenson and Zeiss. Utilizing a carbon arc as source of light, a prismatic system directs a focused beam through the pupil to illuminate the retina. The resultant image is viewed through a reflex lens apparatus to permit fine adjustment of focus. The equipment was portable and was moved to the bedside in each case.

The patient was arranged in a semi-reclining position with his head facing the objective lens at a distance of approximately 10 cm. Application of a few drops of 1 per cent neosynephrin solution insured against pupillary constriction during film exposure. For some patients the lids were taped open and the corneas kept moist by frequent gentle ejections of physiologic saline solution from an ophthalmic irrigator. The carbon arc system was supplied with electric power through a portable rectifier. Limb leads from a portable Burdick direct-writer electrocardiograph were attached to the patient, who was generally unconscious of the proceedings. Each exposure at one eighth of a second on 35 mm. Kodak Ektachrome film was indicated on a continuously running electrocardiographic (EKG) record. Efforts were made to begin photographing the fundus as respirations ceased and to continue to take serial pictures at short intervals thereafter to demonstrate the rapidity with which changes developed.

Results

Six attempts were made; in only 2 cases was the start of the photographic series sufficiently well synchronized with the onset of the patient's death and the resultant serial pictures sufficiently clear to permit complete objective evaluation of the sequence of events.

Case 1

The first patient was a 65-year-old white woman who died of lymphosarcoma a few minutes before the equipment had been set up. The first picture (Fig. 3-A) was taken hastily about 4 minutes after the last EKG oscillation and, consequently, care was not taken to have the disk in view. Arteries had disappeared, but segmented retinal veins persisted. Lacking control pictures of the living fundus, the degree of retinal pallor could not be evaluated. The venous segments appeared to be separated by serum rather than by air, and in some areas fine red streaking could be seen along venous walls in the clear zones. The second picture (Fig. 3-B) was taken 15 minutes after the first and showed essentially the same features (the discoloration was probably an artifact). Movement of segments was not looked for in this case, the first human fundus to be photographed after death in this hospital.

Case 2

A 36-year-old white woman died in coma which had continued for 8 months. She had a huge craniopharyngioma. Again, equipment was not operable at the instant of death. One of four pictures, taken about 25 minutes after respirations ceased, satisfactorily showed a hazily "beaded" vein on a very pale retina devoid of arteries (Fig. 4). Segmentation occurred early in this instance (as observed ophthalmoscopically without EKG control) and was quite remarkable: segments and intersegmentary spaces were short and moved smoothly with a velocity of approximately one to two disk diameters per second in all large venules to the physiologic depression, where they disappeared as over a precipice. Looking like converging trains of boxcars viewed from above, the moving segments presented the most awesome ophthalmoscopic dynamic picture which I have observed. The venous segments continued to move for about 20 minutes after the last breath. Their ends appeared somewhat concave suggesting that the clear spaces in the larger venous tributaries contained compressed gas.

Case 3

A 35-year-old Negress succumbed to metastatic carcinoma of the colon. Faulty adjustment of the carbon light source caused underexposure with totally unsatisfactory photographic results. Ophthalmoscopic examination revealed findings entirely similar to those in case 2.

Case 4

This patient was a 70-year-old white man with massive bilateral cerebral infarcts. The EKG tracing immediately after cessation of respiration (Fig. 1, A-C) showed greatly diminished frequency of heartbeat; a simultaneous photograph (Fig. 5-A) demonstrated hazy granularity, or possibly blurring of moving segments, of arteries only. Veins and retinal color appeared normal (shadow was caused by the untaped upper lid). The photograph reproduced as Figure 5-B, taken by chance just 6 seconds after the last EKG oscillation, revealed arteries disappearing in the direction of flow (the originally "granular" artery was no

longer visible). Venous blood on the now paler and more distinctly outlined disk was irregular in outline due to "granular" flow or moving segments. The entire retina was paler. The subsequent eight serial pictures were ruined by a technical error in exposure. Figure 5-C represents the same fundus approximately 15 minutes after the first picture of the series, but on the opposite side of the disk—a change resulting from hurried rearrangement and repositioning after the error in exposure was discovered. Venous segmentation, though not marked, was then unmistakably evident. Contrary to expectations, the arteries were still apparent; they were thin without "beading." Only in this case of the entire study did such a finding occur. It could not be ascertained whether the arteries refilled after emptying (noted earlier on the other side of the disk), or whether some arteries simply failed to "bead" and disappear.

Case 5

The cause of death of this 59-year-old Negress was esophageal carcinoma with metastases. The photograph (Fig. 7) was taken 15 minutes after clinical death, with no EKG control, and demonstrates remarkably well the venous segmentation with some streaking of erythrocytes between the red segments and, in areas, against the venous walls. The segments continued to move for approximately 12 to 13 minutes after respirations ceased. Light digital pressure upon the globe temporarily reversed direction of the moving segments in some venules, with resumption of the usual flow upon release of pressure.

Case 6

The uremic state of this 51-year-old Negress resulted from chronic pyelonephritis; death occurred immediately after the last of four terminal convulsions. Venous segmentation was observed within seconds after the last EKG activity. Figure 6-A shows indistinct but relatively normal appearing vessels and corresponds to the EKG record of Figure 2-A which showed a frequency of two beats per second. Although electrical heart activity was still present (Fig. 2-B), the frequency of seven beats per minute was too slow to prevent venous segmentation and diminishing caliber of arteries (Fig. 6-B, 45 seconds after 6-A). Figure 6-C represents the same fundus 45 seconds after Figure 6-B; arteries are barely evident.

Case 7

The fundus of a cadaver was examined in the necropsy room 3 hours after death and appeared exactly as depicted by Bouchut[1] (Fig. 8). Merely touching the globe with a finger obliterated the pink-purple venous segments at the disk; removal of the finger resulted in return of segments to exactly their original sizes and positions.

Discussion

Conditions for Successful Ophthalmoscopic Photography

Mechanical accessibility of an adequate amount of light to the retina is the obvious necessary basis of ophthalmoscopy. As a rule pupils dilate somewhat during the process of death, sufficiently to render the routine use of mydriatic solutions unnecessary. In photography with a carbon arc light source, however, iridal light reactions must be prevented. If pupils remain too small at death, e.g., in cases of morphine poisoning, post-mortem use of mydriatics will prove to be effective until iridal rigor occurs in 3 to 4 hours after death.[3]

Clarity of intra-ocular media is another factor of great importance. Extensive, severe, corneal scarring, inflammatory exudation, cataracts, or opacification of the vitreous body during life preclude retinoscopy. Rarely, however, are such obstacles encountered bilaterally; since fundic changes of death are usually similar in both eyes,[8] unilateral examination ordinarily suffices. Frequent digital closure of the lids after death may not maintain corneal moistness and transparency during prolonged examination. The resulting rapidly developing haziness of the corneal surface is best dispelled by applying a drop or two of normal saline solution every 30 to 60 seconds. Plain water has been advocated,[3,10] but protracted use of other than physiologic saline solution may itself eventually cause corneal turbidity. This simple maneuver is *extremely* important, for it allows one to view the retina in very sharp detail for hours, until parenchymal turbidity of the cornea becomes manifest.[8]

Severe, extensive, bilateral intra-ocular diseases also may create insurmountable obstacles to ophthalmoscopy. It already has been mentioned that the usual intra-ocular pathologic changes seldom create confusion with the mortal signs[9]; but large retinal detachments, intrabulbar hemorrhages, large neoplasms, and perhaps unusually severe hemorrhagic exudative retinopathy make ophthalmoscopy impossible.

General Comments

In general, after "EKG death" the arteries are either completely empty or rapidly emptying, presumably in the direction of flow, although evacuation can conceivably take place in both directions simultaneously. The single exception to this usual arterial behavior at death was noted in case 4 of this study. With slowing of heart rate, arterial "granularity" sometimes becomes manifest in the larger branches; that it occurs at all has been questioned by some workers,[6] but not flatly refuted.

Venous irregularity due to marked "granularity" or to moving segments is observed almost invariably within several seconds after cardiac output ceases,[6] as is borne out by the photographs. The first change usually occurs in the large trunks and finer venules on the disk[8] where early segmentation is the rule. Distribution of segments over the fundus varies from case to case[3,23] and some authors insist that this feature may be undetectable early in rare instances.[3,7,10,11,23]

The ultimate cause of venous blood segmentation, a clumping of erythrocytes as circulation loses speed,[2,6] remains obscure. Deceleration and inertia may in themselves be the answer, with post-mortem

equalization of the arterial and venous pressures and a "milking" arteriolar peristalsis accounting for segmental motion[6,23] which is occasionally unapparent in old and debilitated animals.[23] It is interesting to speculate just what rôle, if any, a generalized tendency for body blood to "sludge" during morbid states[24] may play in the genesis of segmentation. Another mechanical factor may be fundamental, a relative increase in intra-ocular pressure. Usher[2] claimed to have caused transitory segmentation during life by injecting saline solution into the eye and, conversely, to have prevented it in animals at death by removing the cornea. It is true that while intra-ocular pressure begins to fall after death,[8] it remains considerably elevated for a time relative to intravascular pressure which is nil; this consideration lends weight to Usher's theory. The possibility of segmentation with glaucoma during life, then, becomes a plausible assumption, but to my knowledge this has not been reported. It may be either that local, intra-ocular, compensatory, vascular pressure changes serve to restore the pressure balance, or that segmentation does occur during late stages of the disease when retinal visualization is no longer practicable.

The only known vital process which produces retinal vascular changes similar to those of generalized circulatory standstill is occlusion of the central retinal artery,[9] either by thrombosis or by embolism. However, the changes under such circumstances tend to occur more slowly, with accompanying edema and hemorrhages. Both causes are rare, and simultaneous bilateral occurrence especially so.

Probably, moderate variations in temperature have no effect upon the appearance of the signs. The results of experiments on hypothermic rabbits strongly negate earlier observations[2] on human corpses. Six anesthetized rabbits with normal rectal temperatures of 39° to 40° C. were subjected to hypothermia. In 3 of them, which evidently froze to death, retinal pallor and venous segmentation with "granularity" were obvious on and around the disk at the time of removal from the bath; rectal temperatures were 14°, 20°, and 21° C., respectively. Arteries had disappeared. Two animals survived shorter exposures, having temperatures of 29° and 32° C. Rapid intravenous injection of calcium chloride solution was followed almost instantaneously in both animals by pallor and segmentation as described. In no instance during this experiment was segmentation as clear-cut as is usual in human eyes, but rather tended to be more "granular" and "sludged" without motility. After 1 hour in the bath, one rabbit with a temperature of 15° C. appeared to be dead on inspection and palpation. Retinal vessels were thinned but continuous. Typical changes of actual

death, however, resulted from a rapid injection of calcium chloride. As a control, one rabbit anesthetized with intravenous pentobarbital was sacrificed by means of intravenous calcium gluconate, without being immersed in an ice bath; segmentation was definite and rapid.

As to the effect of high temperatures, most of the patients photographed had low-grade fevers; and early segmentation in case 6 (Fig. 6-B) is indisputable in spite of excessive hyperpyrexia at death (over 107° F., rectally). It seems reasonable to conclude that the usual behavior of retinal vessels when heart output stops is not modified by variations in body or environmental temperatures within relatively wide range.

In conclusion, these rapid signs—above all, "granularity" and segmentation of columns of venous blood—signify only one thing: *absence of cardiac output,* due either to a weak, non-functional beat, or to complete arrest, ventricular fibrillation, or complete heart block (patients with Stokes-Adams syndrome could conceivably show the signs).[6] When seen, they indicate cessation of blood flow to the eyes and therefore cessation of flow to the brain, and, obviously, complete cessation of flow to all organs—or practical death. It is this period of terminal agony (vascular narrowing and "granularity") and so-called clinical death (segmentation *with* movement) which is of utmost importance in resuscitable cases.[25] No known clinical or laboratory aid other than the ophthalmoscope can determine so quickly whether circulation has stopped recently enough to allow for resuscitation without severe residual central nervous system disintegration: the electrocardioscope[25] and the EKG indicate merely that flow or heart action has stopped, with no indication as to elapsed time. The EKG may, in addition, show normal or abnormal oscillations (injury currents) up to an hour or more after complete standstill.[26] If venous segmentation with movement is seen ophthalmoscopically, it is reasonable to assume that cardiac standstill could have been no more than 10 to 20 minutes antecedent to examination, and vigorous resuscitative measures should be attempted. After segmental movement has stopped, resuscitation is virtually hopeless despite any undertaking.[6]

In this connection, Wegner's work[27] (1933) is of special interest. He determined that the retina can endure complete occlusion of its blood supply for 22 minutes without suffering any permanent functional impairment after restoration of flow. The critical time range in which cells began to disintegrate, producing visual impairment, was from 22 to 45 minutes. After cardiac standstill the venous segments continue to move as long as 20 ± minutes. This may serve as an indi-

cation not only of the possibility of retinal restitution, but also that similar vascular phenomena in other, invisible organs of the body, including the heart, may maintain the entire organism in a resuscitable state for that period of time.

The ophthalmoscopic signs may find useful application as follows:

1. In general clinical practice: to determine a state of death; to differentiate between real and apparent death and to determine resuscitability of victims of fainting, pathologic sleep, electrocution, deep coma, profound shock; narcosis (barbiturates, chloroform), asphyxiation, or drowning; to differentiate real shock from cardiac arrest or fibrillation during surgery; and to determine death in patients in respirators.

2. In forensic medicine: to estimate time of death in minutes (moving segments) or hours (static segments and retinal color); and to determine the position of the head at death.

Summary

There are ophthalmoscopically observable changes in the color of the retina and in the caliber and content of the retinal vessels which are indicative of impending and of actual death. Of special importance are "granularity" of venous blood and of moving or static segmentation of the columns of red blood cells in the veins. These changes are illustrated by colored photographs which are synchronized (for the first time) with electrocardiographic tracings.

These changes provide a readily available means of determining the fact of death, of differentiating apparent and actual death, of estimating (within a certain range) the elapsed time after death, and, of great importance, of indicating the possibility of success from efforts at resuscitation.

I am indebted to Dr. Conrad S. Heyner of the Department of Ophthalmology, University of Michigan, for assistance and encouragement throughout this project; and for the loan of the photographic apparatus.

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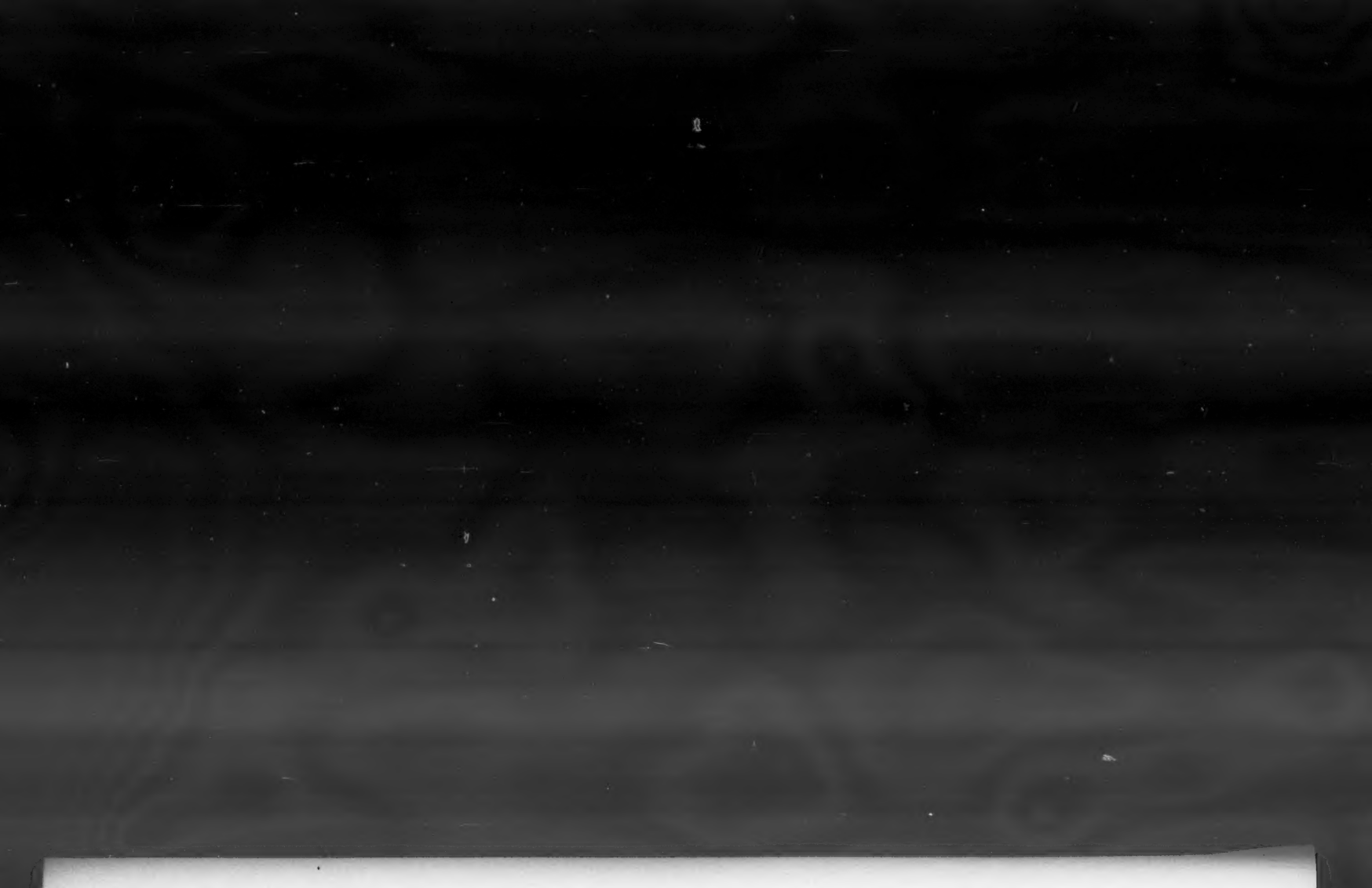
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. EKG tracings in case 4. *A*. Several minutes before respirations ceased. Triangles delimit an interval of 1 second. *B*. Approximately 45 seconds after respirations ceased. Although the exact time was not indicated, Figure 5-A was taken during this period. *C*. The last EKG oscillation. Arrow indicates time of Figure 5-B, approximately 30 to 40 seconds after Figure 5-A.

FIG. 2. EKG tracings in case 6. *A*. Approximately 3 hours before death; 1 second interval between triangles. This was the EKG pattern with Figure 6-A. *B*. Immediately after the final convulsion; respirations were undetectable. Arrow marks point at which Figure 6-B was taken. *C*. The last two EKG oscillations. Arrow shows when Figure 6-C was taken, 45 seconds after Figure 6-B.



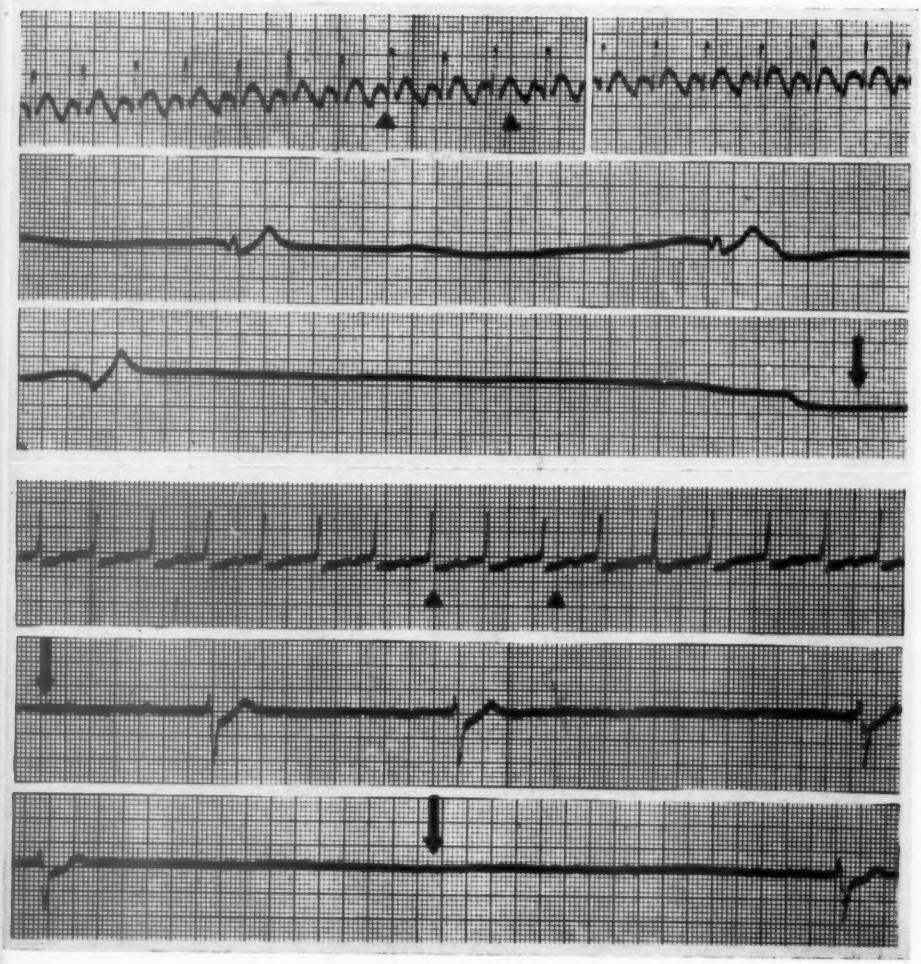

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FIG. 3. Fundus in case 1. *A*. Approximately 4 minutes after "EKG death." *B*. Fifteen minutes after A.

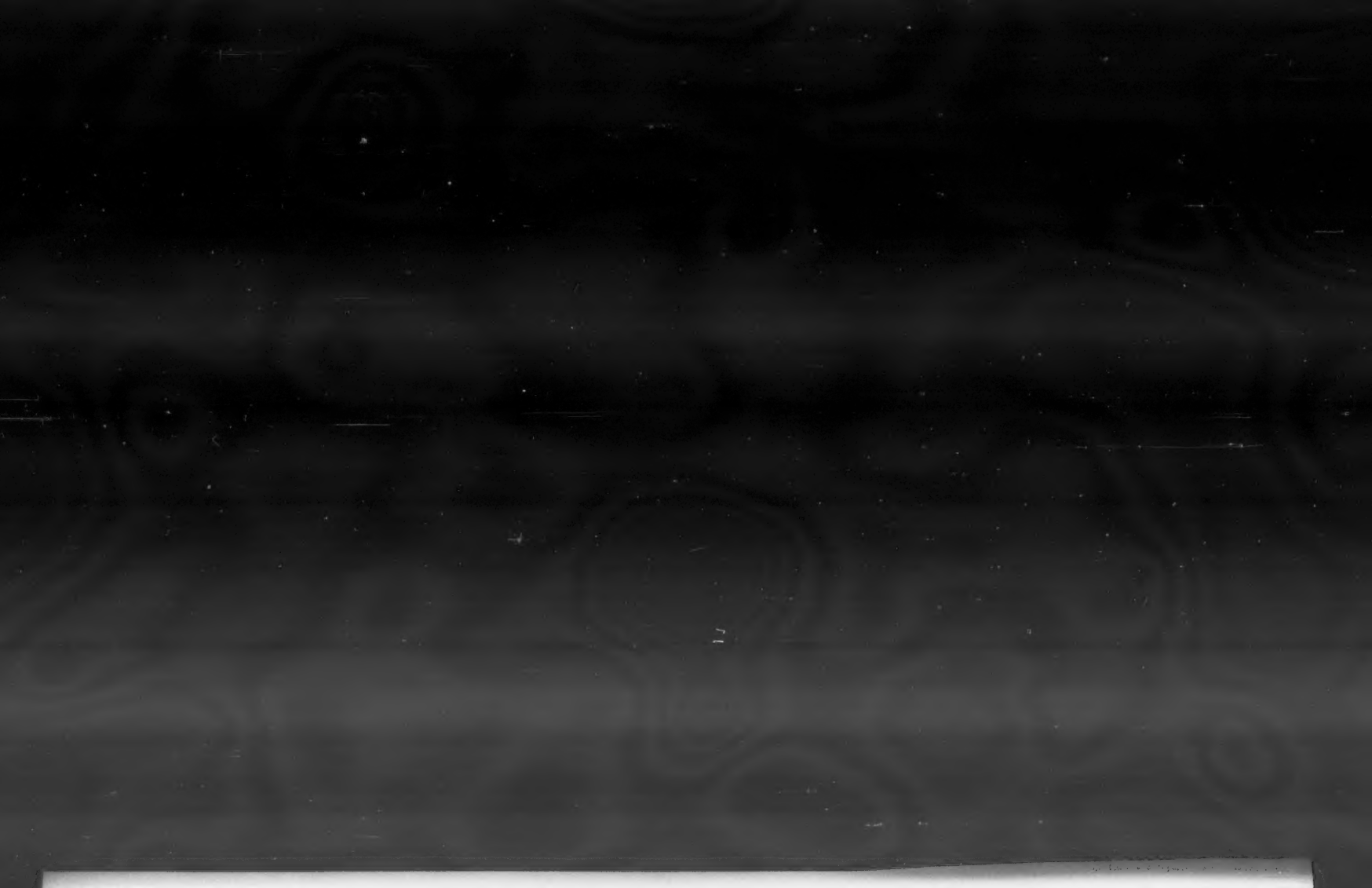
FIG. 4. Fundus in case 2. Approximately 25 minutes after respirations ceased.

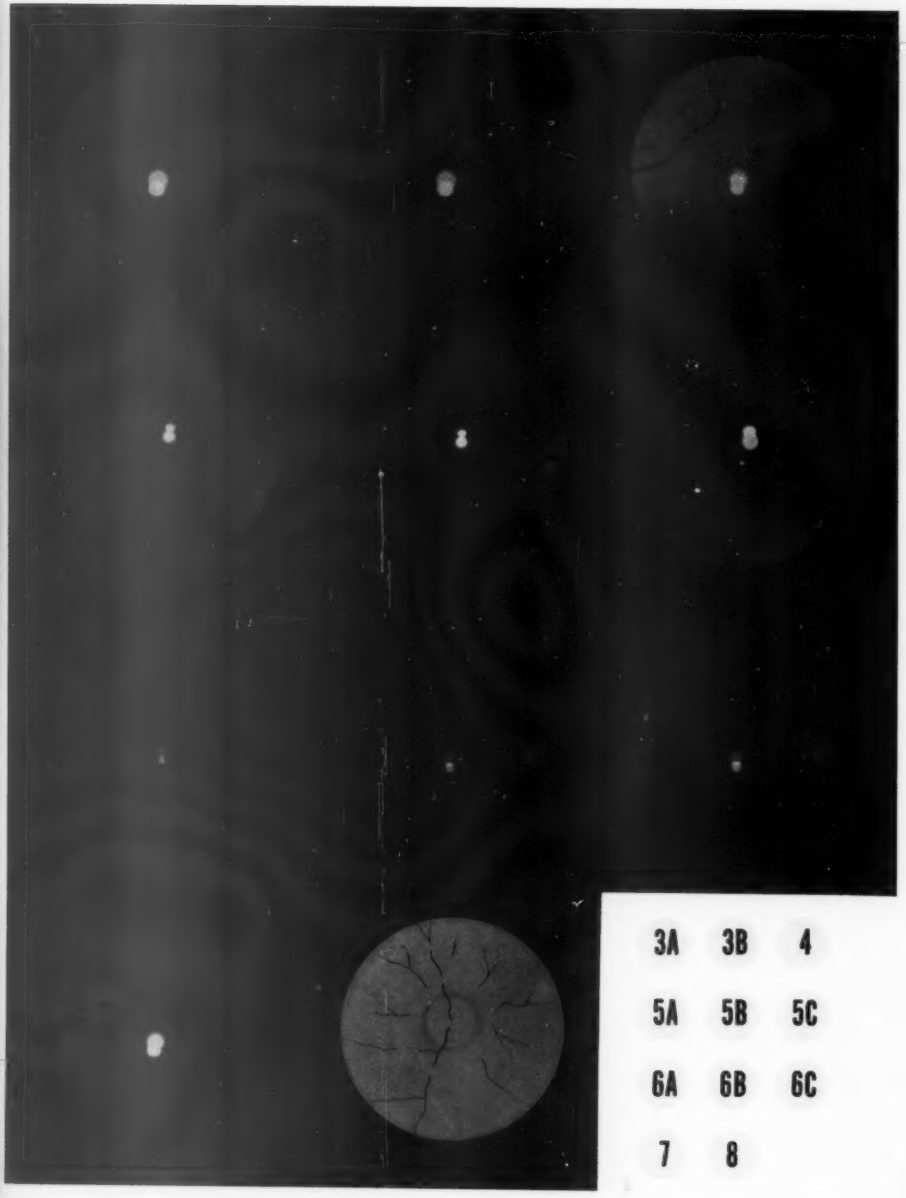
FIG. 5. Fundus in case 4. *A*. Approximately 45 seconds after respirations ceased. EKG frequency about 15 oscillations per minute (see Fig. 1-B). *B*. Same fundus 30 to 40 seconds later, 6 seconds after last EKG oscillation (see Fig. 1-C). *C*. Same fundus 15 minutes later, opposite side of disk.

FIG. 6. Fundus in case 6. *A*. Approximately 3 hours before death (see Fig. 2-A). *B*. Same fundus and generally the same site with the disk now visible. Respirations had stopped; EKG frequency very low (see Fig. 2-B). *C*. Same fundus 45 seconds later, at the time of "EKG death" (Fig. 2-C).

FIG. 7. Fundus in case 5. Fifteen minutes after clinical death.

FIG. 8. Photographic reproduction of Bouchut's original drawing.[1]

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